Investigation of the cellular tropism and *in vivo* distribution of HIV-1

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Declaration

The results presented in this thesis and its composition were undertaken solely by the author unless otherwise stated.

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Abbreviations

3TC	Lamivudine
ADC	AIDS dementia complex
AIDS	Aquired Immunodeficiency Syndrome
AZT	Zidovudine
BBB	Blood brain barrier
BSA	Bovine Serum Albumin
C1-C5	Conserved regions of gp120
CA	Capsid
CAF	CD8 anti-HIV factor
CNS	Central nervous system
CR1	Complement receptor 1
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
d4T	Stavudine
DAB	Diaminobenzidine
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific inter cellular adhesion molecule-grabbing nonintegrin
DMEM	Dulbecco's modified eagle's medium
dNTP	Deoxynucleoside triphosphates
ECF	Extra-cellular fluid
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
Env	Envelope
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FIV	Feline Immunodeficiency Virus
Gag	Group specific antigens
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent protein
gp120	Surface portion of Env
gp41	Transmembrane portion of Env
HA	Haemagglutinin
HAART	Highly active anti-retroviral treatment
HAD	HIV-associated dementia
HBSS	Hank's buffered saline solution
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HIVE	HIV encephalitis
HLA	Human Leukocyte Antigen
IFN	Interferon
IN	Integrase
JC	Jukes cantor
LB	Luria-Bertani
LTNP	Long term non-progressors
LTR	Long terminal repeat

MA	Matrix
mAb	Monoclonal antibody
MBL	Mannose binding lectin
MENI	Multiply exposed non-infected
MHC	Major histocompatibility complex
NC	Nucleocapsid
Nef	Negative infectivity factor
NES	Nuclear export signal
NLS	Nuclear localization signal
NMS	Normal mouse serum
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside-analogue reverse transcriptase inhibitor
NSI	Non-syncytium inducing
ORF	Open reading frame
P.t.t	Pan troglodytes troglodytes
p24	p24 antigen also known as capsid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
PI	Protease inhibitor
PIC	Pre-Integration Complex
PR	Protease
R5	CCR5 using virus
Rev	Regulator of viral expression
RPMI	Roswell Park Memorial Institute media
RRE	Rev responsive element
RT	Reverse transcriptase
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SHIV	SIV/HIV chimeric virus
SI	Syncytium inducing
SIV	Simian Immunodeficiency Virus
Taq	*Thermus aquaticus*
TAR	Transactivation response region
Tat	Transactivator of transcription
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
X4	CXCR4 using virus
X4R5	Dual tropic virus

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Abstract

Since its discovery in 1983, HIV-1 has spread rapidly resulting in a global pandemic. Currently an estimated 40 million people are infected with the virus. Infection leads eventually to the acquired immunodeficiency syndrome (AIDS) and death. The symptoms of the syndrome are largely due to the devastating effect HIV has on the immune system. HIV has been the focus of an unprecedented amount of research however there are still many aspects of HIV pathogenesis which remain incompletely characterized. One area which continues to attract attention is HIV tropism. HIV requires two receptors- CD4 and a co-receptor- for infection. *In vivo*, HIV variants mainly use either CCR5 or CXCR4 as co-receptors and thus variants can be classified as either R5 (CCR5 using), X4 (CXCR4 using) or (R5X4) (dual tropic). However these classifications do not fully explain the differing abilities of variants to infect particular cell types. It is thought that other factors, such as receptor density or alternative receptors, may underlie observed differences in tropism. In addition to studying viral variants to investigate determinants of tropism, it is also possible to investigate post-mortem material from HIV infected individuals to characterize nature and cellular location of HIV variants. HIV can infect cells in the brain and this leads in some cases to HIV associated dementia (HAD). The presence of HIV in the brain poses a challenge to attempts to eradicate the virus as many drugs have poor CNS penetration. There have been concerns that sub-optimal levels of drugs in the CNS may support the evolution of drug resistant variants. Alternatively, low levels of drugs may allow growth of drug sensitive variants which could reseed the periphery if the drug regime was halted. Characterisation of the provirus present in post mortem brain material could lead to a better understanding of this issue. The virus in the brain appears to mainly infect microglia and macrophages however it is not clear to what extent other brain cells might be infected. Conflicting reports have emerged regarding the infection of astrocytes and neurons. Thus a method for separating the various brain cells which allows them to be tested for HIV could be valuable.

In the first part of this project, methods to characterise the cellular tropism of HIV were investigated. A problem with many previous methods is that manipulation or amplification of the HIV genome has resulted in artefactual changes in the sequence. Thus any results obtained regarding the cellular tropism of variants may not accurately reflect the *in vivo* situation. A vector was produced which could be used to produce recombinant HIV expressing study subject derived *env*. The vector incorporated the EGFP to allow easy identification of infected cells. Three methods for production of virus without the need to amplify the genome in *E. coli* were designed. All methods were capable of producing infectious virus expressing various *env* genes.

The second part of the project sought to investigate the distribution of drug resistance variants *in vivo*. DNA was extracted from post mortem brain and lymphoid tissues from HIV infected study subjects. Limiting dilution PCR was used to obtain PCR products each derived from a single template molecule. No differences were found in the distribution of drug-resistance mutations between the two compartments for three out of four study subjects. In one study subject there were considerably more drug resistance mutations in the lymphoid derived sequences than in those amplified from brain tissue. This is probably due to low levels of anti-HIV drugs in the CNS of this subject.

In the final part of the project, a method for the bulk isolation of microglia and astrocytes from post-mortem HIV-infected brains was optimised. Cellular debris was first removed using a density gradient. The cells were then fixed using a gentle ethanol fixation technique. This inactivated the HIV, fixed and permeabilised the cells while maintaining DNA integrity. FACS sorting was carried out using CD68 and GFAP as markers of microglia and astrocytes respectively. Separation of cells was carried out using two HIV-infected brains. No provirus was detected in the separated cells however the brain material used was from asymptomatic study subjects and had extremely low proviral loads. It is hoped that in the future the technique could be used to separate brain material which has a higher proviral load. This would allow quantification of the level of HIV infection of these two cell types.

Chapter 1: General Introduction

1.1 Background

1.1.1 Identification of AIDS

The syndrome now known as the acquired immunodeficiency syndrome (AIDS) was first noticed by physicians in the United States in the late seventies and early eighties. A report in Morbidity and Mortality in 1981 described five previously healthy young Californian men who had recently developed lymphadenopathy, lowered CD4 T cell counts along with diseases consistent with a lowered immune response (CDC, 1981). These included infections such as *Pneumocystis carinii* pneumonia, *Toxoplasma gondii* encephalitis, cytomegalovirus-associated retinitis and cryptococcal meningitis as well as unusual cancers such as non-Hodgkins lymphoma and Kaposi's sarcoma. This publication prompted a number of other physicians who had observed similar clinical pictures to come forward (Masur et al., 1981; Siegal et al., 1981; Gottlieb et al., 1981). With increasing reports from each centre, it was noted that the syndrome mainly affected certain groups of people; men who have sex with men, intravenous drug users, haemophiliacs, blood-transfusion recipients, recent Haitian immigrants and the sexual partners of any of the above. This epidemiological pattern strongly suggested that the syndrome was caused by a pathogen which could be transmitted in infected blood or by sexual intercourse with an infected person.

The etiological agent of AIDS was first identified by Barre-Sinoussi and colleagues in 1983 (Barre Sinoussi et al., 1983). These researchers were able to culture a virus from a lymph node biopsy obtained from an individual with AIDS. Reverse transcriptase was identified in the culture supernatant

confirming that a retrovirus was present. Subsequent studies by two groups in the USA provided further evidence that a new retrovirus was the causative agent of AIDS (Gallo et al., 1984; Levy et al., 1984). Initially each group assigned the virus a different name: lymphadonopathy-associated virus (LAV), human T-cell lymphotropic virus III (HTLV-III) and AIDS-associated retrovirus (ARV) respectively. However it became clear that these viruses shared many common features and in 1986, the International Committee for the Taxonomy of Viruses named the virus the human immunodeficiency virus (HIV) and later HIV type 1 (HIV-1) (Coffin et al., 1986). Also in 1986 a second human immunodeficiency virus was isolated from individuals in western Africa. This virus was genetically distinct from and less pathogenic than HIV-1 and was termed HIV type 2 (HIV-2)(Clavel et al., 1986b; Clavel et al., 1986a).

1.1.2 Origin of HIV

Both HIV-1 and HIV-2 are thought to have arisen by the transfer of distinct Simian Immunodeficiency Viruses (SIVs) into the human population (reviewed in McGrath et al 2001). It is thought that cross-species infection probably first occurred into persons butchering monkey meat.

The source of HIV-2 was identified as the sooty mangabey monkey found in West Africa (Gao et al., 1992; Hirsch et al., 1989). Up to 30% of wild-caught sooty mangabeys harbour SIVsm (Sharp and Li, 1988). Phylogenetic analysis indicates that SIV_{SM} and HIV-2 are interspersed. This suggests that multiple cross-species transmissions have occurred. The source of HIV-1 is the chimpanzee species *Pan troglodytes troglodytes* (*P.t.t*) (Gao et al., 1999).

A number of strains of SIVcpz have been isolated from *P.t.t.* and these show significant similarity to HIV-1. There are three genetically distinct groups of HIV-1 viruses: group M (main) is the group responsible for the current global pandemic. Groups O (outlier) and N (non-M, non-O) have only resulted in a small number of infections. It is assumed that HIV-1 groups M, N and O were initiated by three separate cross-species transmissions. Group M viruses are further divided into various subtypes (figure 1.1).

The timing of the crossover to human recipients of the ancestor of HIV-1 group M has been estimated using computational phylogenetic analysis (Korber et al., 2000). According to this analysis, the most recent common ancestor of HIV group M occurred around 1931. The earliest known sample of HIV dates from 1959 (Zhu et al., 1998). The computational method used by Korber et al was validated by correctly estimating the date of this, and another historical sequence.

1.1.3 Transmission

HIV can be transmitted in a variety of ways but the vast majority of infections are acquired through sexual contact (reviewed in Cohen and Fauci, 2001). While the epidemic was first noticed in men who have sex with men, the majority of infections worldwide occur through heterosexual contact. The per contact risk of becoming infected as a result of sex with an infected partner is low; most estimates are less than 1% (Hansasuta and Rowland-Jones, 2001). The precise risk varies considerably depending on a number of factors including type of sexual contact, genetic factors, presence of genital ulcers or sores and size of inoculum.

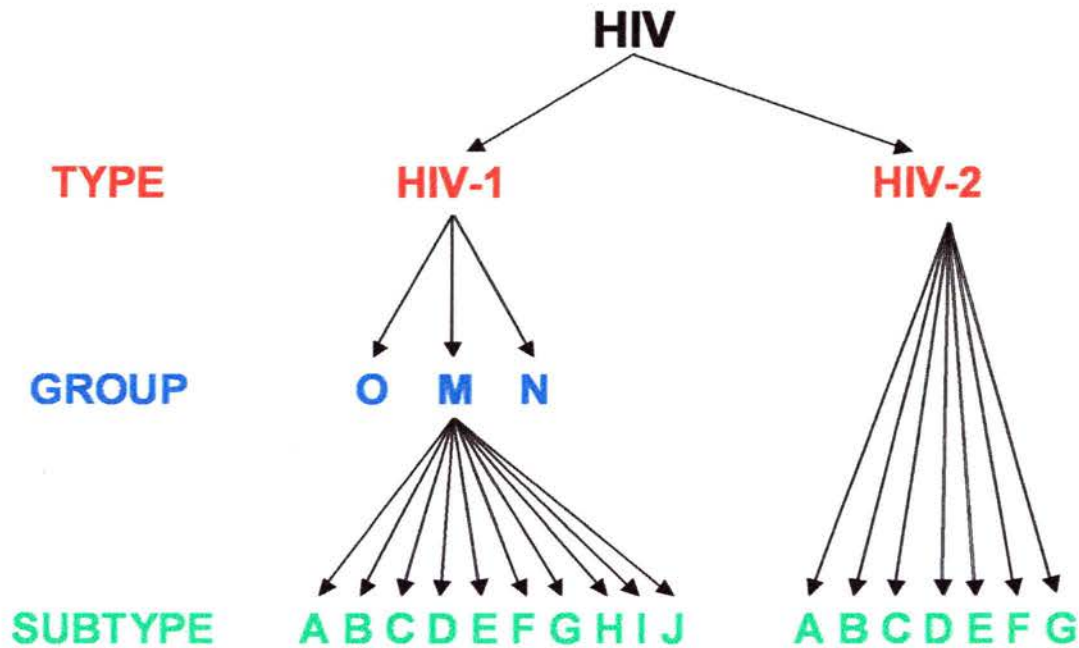


Figure 1.1: Classification of the human immunodeficiency viruses. Circulating recombinant forms are not shown on this figure.

Tragically, HIV can be transmitted to babies during birth or via breast feeding. In some poorer countries, as many as 42% of children born to infected mothers become infected (Datta et al., 1994).

HIV can be transmitted in infected blood products or by shared use of contaminated needles. A large number of haemophiliacs were infected due to HIV infected blood transfusions in the 1980s (reviewed in Levine, 1985). Epidemics have also occurred in populations of intra-venous drug users where needle-sharing is common. In a worldwide context, needle-sharing is a relatively minor mode of transmission however, in some areas, including Edinburgh, this is the main source of new HIV infections (Abdala et al., 2003; Bell et al., 2002).

1.1.4 Clinical features

There are three phases of HIV infection each with quite different clinical pictures (reviewed in Cohen and Fauci, 2001). During acute infection, up to 90% of individuals experience a flu-like illness. Symptoms may include fever, fatigue, headache, vomiting, diarrhoea, myalgia and a cutaneous rash. Due to the non-specific nature of these symptoms, HIV infection is not usually recognised at this point. In one study only 25% of people presenting to a clinic with acute infection were diagnosed with HIV infection at the time (Schacker et al., 1996). After this acute episode, individuals enter an extended period (approximately 10 years) of clinical latency. In this period the immune system is able to function fairly well although there may be some signs of mild immunosupression especially as the CD4 T cell count falls below 500 cells/µl. Symptoms may include fatigue, lymphadonopathy,

prolonged and atypical infections and mild neuropathy. The diagnosis of AIDS is made in an HIV infected individual when the CD4 T cell count falls below 200 cells/μl or if the person displays a clinical picture consistent with severe immunodeficiency. This state is characterised by so called "opportunistic infections". These are caused by organisms which are non-pathogenic to healthy individuals but are able to cause severe disease in immuno-compromised individuals. In addition a number of malignancies can arise in AIDS. These may also be initiated by infectious organisms.

1.2 Pathogenesis

The immunodeficiency seen in AIDS is due to a decrease in T cell numbers and probably also aberrant T-cell function. Figure 1.2 shows how T cell numbers and viral concentration vary over the course of HIV infection.

1.2.1 Mechanisms of cell death

Most T cells which die during HIV infection do so by apoptosis (reviewed in Alimonti et al., 2003 and Gougeon, 2003). There are three main pathways which lead to cell apoptosis in HIV infected individuals: apoptosis of HIV infected cells; activation induced apoptosis and HIV protein induced apoptosis. The latter two occur mainly in non-HIV infected cells. The relative contribution of each pathway to overall pathogenesis remains controversial and it is likely to vary in different stages of disease. In particular, apoptosis of infected cells is thought to play a minor role in T cell depletion during chronic HIV infection while it may be a major factor in acute infection.

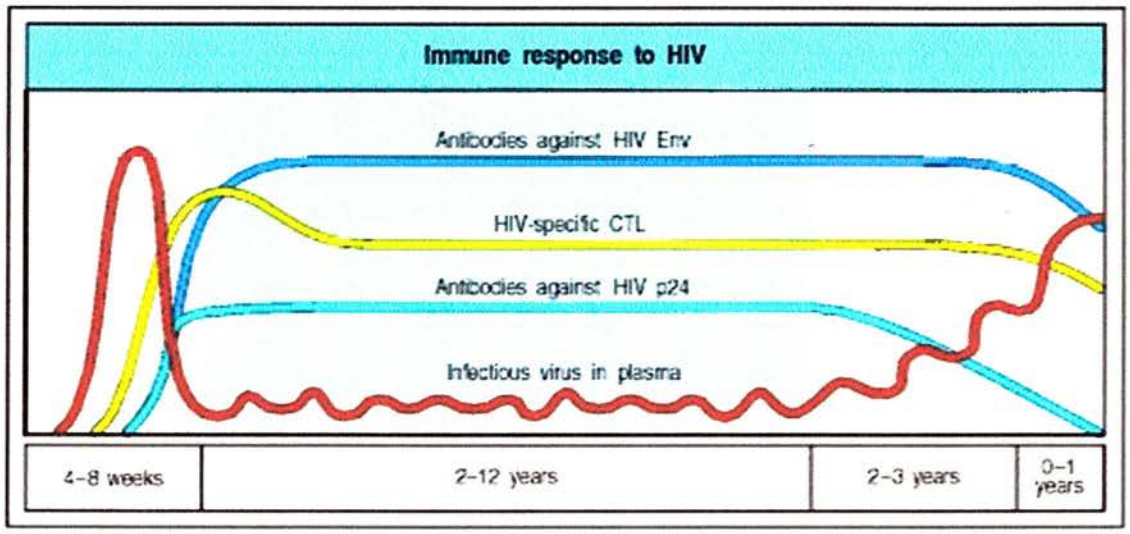


Figure 1.2: T cell and viral dynamics over course of HIV infection (Janeway 2001).

HIV has a number of methods of promoting apoptosis in infected cells. The host cell membrane can be disrupted by continual viral budding (Fauci, 1988) or by the permeabilising effect of Vpu (Gonzalez and Carrasco, 2001). Build up of unintegrated proviral DNA can be toxic (Shaw et al., 1984). HIV proteins are also able to upregulate expression of pro-apoptotic genes or down-regulate anti-apoptotic genes in infected host cell (Alimonti et al, 2003). Surprisingly, other HIV proteins act to inhibit apoptosis of infected cells. Nef and Tat mediated down-regulation of MHC I prevents CTL-mediated killing (Alimonti et al, 2003). Vpr appears to have a role in upregulating an anti-apoptotic and decreasing a pro-apoptotic protein (Conti et al., 1998). It is thought that the combination of these effects may be to prolong cell survival long enough to produce new infectious virus while maintaining a reduced half-life compared with uninfected cells.

Activation-induced cell death is a normal physiological phenomenon by which the immune system prevents excess proliferation of T cells. Activation itself, up-regulates factors such as Fas and Fas ligand, which later induce apoptosis of the cell (Lenardo et al., 1999). HIV indirectly causes massive amounts of cell death simply by activating T cells which then undergo apoptosis.

A number of HIV proteins when released into the extra-cellular environment can induce apoptosis in uninfected cells. Released gp120 can cause apoptosis of nearby cells via interaction with CD4 and co-receptor molecules (Arthos et al., 2002). In addition, infected cells displaying gp120 on their surface can induce apoptosis of neighbouring cells (Blanco et al., 2003).

Extracellular Tat can be endocytosed by cells. Once in the cell, Tat has a number of effects which act to promote apoptosis (Li et al., 1995). It is also thought that Tat in infected monocytes acts to upregulate TNF-related apoptosis-inducing ligand which through interaction with nearby cells promotes bystander apoptosis (Zhang et al., 2001). Finally, Nef and Vpu are able to cause membrane disruption of both CD4+ and CD4- cells (Azad, 2000).

1.2.2 Initiating and propagating infection

It has been impossible to study the events immediately post HIV infection in humans. There have, however, been numerous studies of mucosal infection of rhesus macaques with $SIV_{MAC}251$ (reviewed in Weber, 2001; Douek et al., 2003) or studies of HIV infection of tissue culture cells. It is assumed that early events in HIV infected humans may mirror those observed in these models. After mucosal infection it appears that the major site of viral replication is the population of CD4 T cells resident in the lamina propria of mucosal tissues (Veazey et al., 1998; Stahlhennig et al., 1999).

Another cell type which may play an important role in early infection are dendritic cells (DC) (reviewed in Steinman et al., 2003). Blood monocytes which have been induced to differentiate into immature DCs ex vivo can be productively infected with HIV as can skin-derived Langherhan's cells. However, infection levels in both of these *in vitro* experiments have been low (less than 10%). There are conflicting reports regarding productive infection of DCs *in vivo* (Tenner-Racz et al., 1994; McIlroy et al., 1995). Current consensus appears to be that infection of DCs can occur but is extremely

rare.

Perhaps a more significant role for DCs in HIV pathogenesis is their ability to promote T cell infection. When DCs are exposed to HIV and then co-cultured with T cells, substantial productive infection of the T cells occurs (Cameron et al., 1992). It is thought that this *in vitro* phenomenon may mirror the situation in mucosal membranes; DCs could encounter HIV at the mucosal surface, migrate to lymph nodes and then mediate T cell infection. The presence of the C-type lectin DC-SIGN appears to be crucial to DC's ability to transmit infection to T cells (Geijtenbeek et al., 2000). This molecule has an affinity for HIV even higher than that of the CD4molecule (Curtis et al., 1992). It is thought that after binding, DC-SIGN internalises the virus thus protecting it during the journey to the lymph node. In the lymph node the DC can mediate prolific infection of T cells. The mechanism by which DC-SIGN promotes efficient T cell infection is unclear. One theory is that binding to DC-SIGN causes a conformational change in gp120 which increases its affinity for either CD4 or its coreceptor (Geijtenbeek and van Kooyk, 2003). Some DC subsets, such as Langherhan's cells, do not express DC-SIGN and in these some other receptor may mediate HIV uptake and enhance T cell infection. Surprisingly, the presence of a suitable co-receptor on both DCs and T cells is also necessary for efficient transmission of infection (Granelli-Piperno et al., 1998). This suggests that infection of the DCs may play some role in this process.

Other cell types may also play a role in transporting and trapping HIV *in vivo.* Erythrocytes can bind complement fragments in HIV-containing immune

complexes via their complement receptor 1 (CR1) and transmit the infectious virus to susceptible cells (Montefiori et al., 1994; Moir et al., 2000). A significant portion of the viral population can be found in association with erythrocytes (Hess et al., 2002). Follicular dendritic cells can also trap HIV in immune complexes bound to complement receptors (Joling et al., 1993). These cells can maintain HIV in an infectious form for many months and thus may be an important sanctuary site for virus (Smith et al., 2001).

In addition to their role in attachment of HIV, complement components may also enhance HIV infection of some cells (Reisinger et al., 1990; Boyer et al., 1991). Cell lines infected with C3b-opsonised virus showed a significant increase in viral production compared to those infected with non-opsonised virus. This phenomenon has been observed with both CD4 positive and CD4 negative cell lines . The significance of these findings to the *in vivo* pathogenesis of HIV infection remains unresolved.

In macaque model systems, over the first few weeks of infection, there is explosive replication of SIV in CD4 cells in lymphoid tissues including the lymph nodes, spleen, thymus and mucosal tracts (reviewed in Granelli-Piperno et al, 1998; Douek et al, 2003). Limited studies of lymph node biopsies from humans recently infected with HIV have also found that CD4 T cells are the major location of virus (Zhang et al., 1999; Schacker et al., 2001). In particular, CCR5+ CD4+ memory cells are targeted by the virus. The magnitude of this initial burst of replication may be underestimated in studies which only measure CD4 cell loss in the periphery as the majority of T cells are located at mucosal sites. The gastrointestinal tract alone is

thought to harbour around 60% of T cells. Unlike the situation in the blood, lymph nodes and spleen, most T cells found at mucosal sites are of a CCR5+ CD4+ memory phenotype- the main target of acute viral infection. In rhesus macaques Douek et al (2003), reported a modest drop of peripheral CD4 T cells counts following SIV infection while in contrast, CD4 cells at a mucosal site (measured by pulmonary lavage) were almost completely eliminated. In fact, when levels of memory cells, as opposed to total T cell number, were measured in the periphery, a profound depletion was observed. Studies in humans have also revealed a profound loss in intestinal T cell numbers following acute infection which persists throughout the infection (Lim et al., 1993). This dramatic decline is despite a homeostatic increase in proliferation of memory T cells.

It is presumed that the killing of T cells during the acute response is due to direct cytopathic effects of the virus, unopposed as yet by the immune response. Within a few weeks a cell mediated response to the virus has developed. It is thought that this cell mediated response (Koup et al., 1994; Borrow et al., 1994), along with HIV-specific antibodies (Cheingsong Popov et al., 1991), brings to an end the high viraemia seen in acute infection. Another contributing factor may be that the HIV simply runs out of target cells in the mucosa (Phillips, 1996).

1.2.3 T cell dynamics in chronic infection

In order to understand the T cell dynamics of chronic HIV infection it is helpful to study T cell maturation and renewal in healthy individuals (reviewed in Douek et al, 2003; Clark et al., 1999). Both CD4 and CD8 T cells can be

divided into three phenotypically distinct populations. Confusingly, a number of different terms have been used to describe these populations. Here, they will be referred to as naïve, memory and active. These types can be identified experimentally by differential expression of surface markers. Once a naïve cell has been activated by recognising its antigen in the context of MHC and receiving necessary co-stimulation, it can remain active or "fall back" to a memory (resting) phenotype. The precise dynamics of these processes differ between CD4 and CD8 cells. Renewal of the naïve CD4 population is dependent on the thymus while CD8 cells can be produced in both thymus-dependent and thymus-independent manners. Renewal of naïve CD4 cells is slow and it is thought that it occurs at close to maximum capacity even in immuno-competent adults. Once activated the vast majority of T cells die. The portion which does survive and re-enter the memory pool, is far greater for CD8 cells than for CD4s.

Numerous studies have found an increased turnover of both CD4 and CD8 T cells during chronic HIV infection (reviewed in Douek et al, 2003; Clark et al, 1999). It appears that this increase in turnover is mainly in the memory and active populations. These populations are stimulated to proliferate by HIV-induced activation and the natural consequence of activation is subsequent death of most cells.

This process alone does not account for the on-going T cell depletion observed in HIV infected individuals. HIV causes proliferation of activated cells which then die but these cells are programmed to die anyway so this does not drastically affect overall T cell numbers. It is thought that CD4

depletion is rather caused by a more subtle "chipping away" of the immune system's renewal mechanisms. There is chronic activation and differentiation of both the naïve and memory populations (Grossman et al., 2002). The population of memory cells has already been severely depleted during the acute phase of infection and cytopathic effects of the virus may continue to play a role in killing CD4 cells. In addition the capacity of the bone marrow and thymus to renew these populations is severely impaired by HIV infection (Mir et al., 1989; Gaulton et al., 1997). Over time the combination of these effects causes a gradual decline in CD4 T cell numbers eventually leading to outright immuno-suppression. The ability of the CD8 cell population to remain constant through much of the infection is probably due to its distinct renewal mechanisms.

1.3 HIV

1.3.1 Virion Structure

HIV is a member of the lentivirus genus made up of retroviruses with complex genomes and a cone-shaped nucleocapsid. HIV particles are spheres of approximately 100nm diameter (figure 1.3) (reviewed in Freed and Martin, 2001; Turner and Summers, 1999). The outermost layer of all lentiviruses is a lipid membrane derived from the host cell during viral budding. Embedded in the membrane are various host membrane proteins including major histocompatibility antigens, actin and ubiquitin (Arthur et al., 1992). In addition the HIV envelope (Env) protein is found protruding from the membrane. Attached to the inner surface of this membrane via interactions with Env is the matrix shell consisting of around 2000 copies of the matrix

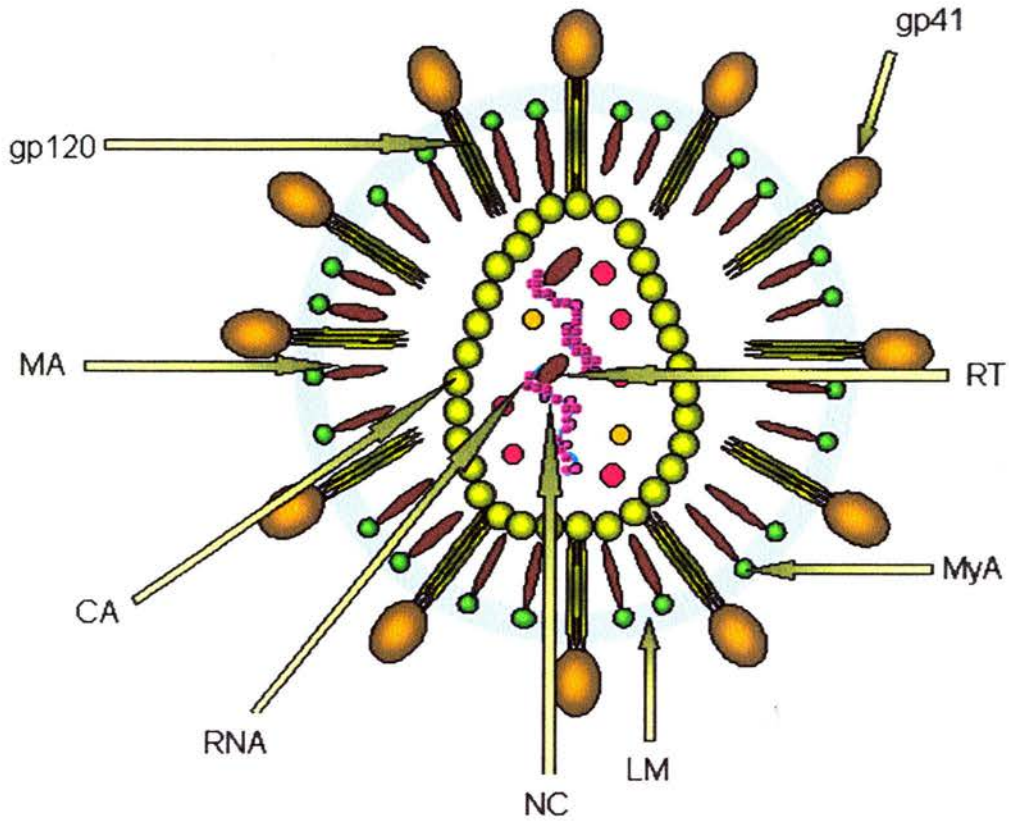


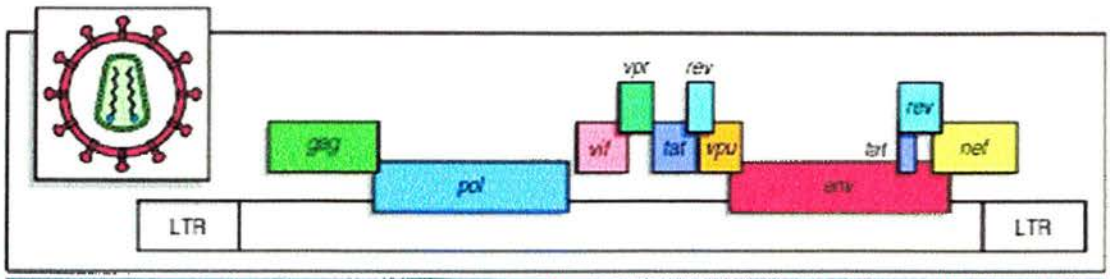
Figure 1.3: Schematic representation of HIV virion structure showing location of various components. See text for more details. MA: Matrix; CA: Capsid; NC: Nucleocapsid; LM: Lipid Membrane; MyA: Myristic acid moity; RT: Reverse Transcriptase (Adapted from Hunt 1999).

(MA) protein. Likewise, around 2000 copies of the capsid (CA) protein make up the cone-shaped inner capsid. Within this compartment are the two copies of the viral RNA genome stabilised by an interaction with around 2000 copies of the nucleocapsid (NC) protein. Also within the capsid are the viral enzymes (integrase (IN), reverse transcriptase (RT) and protease (PR)), viral protein R (Vpr) and the p6 protein. Host cell-derived cyclophilin A is incorporated via its interaction with CA. Viral proteins Nef and Vif are also incorporated although the significance of this is unclear.

The genomes of all retroviruses possess three genes known as *gag*, *pol* and *env* (reviewed in Freed and Martin, 2001). When the HIV genome was first investigated it caused surprise because, unlike the simple retroviruses previously identified, it contained a number of additional overlapping genes (figure 1.4). The open reading frames for some of these are split by DNA segments and thus RNA splicing is necessary for their expression.

1.3.2 Genetic diversity and distribution

HIV-1 group M viruses have been subdivided into subtypes according to phylogenetic analysis of *gag* and *env* sequences. So far nine subtypes and ten circulating intersubtype recombinant forms (CRFs) have been identified (Tatt et al., 2001). The various subtypes and CRFs have heterogeneous distributions and disease impacts (figure 1.5) (reviewed in Tatt et al, 2001 and Essex, 1999). Over half of infections are caused by subtype C which is the main strain in Southern Africa, India and China. Subtypes A and D are the main strains in more northern areas of sub-Saharan Africa. In South-East Asia there is epidemic spread of CRFA_E. In North America and Western



Gene		Gene product/function
gag	Group-specific antigen	Core proteins and matrix proteins
pol	Polymerase	Reverse transcriptase, protease, and integrase enzymes
env	Envelope	Transmembrane glycoproteins: gp120 binds CD4 and CCR5; gp41 is required for virus fusion and internalization
tat	Transactivator	Positive regulator of transcription
rev	Regulator of viral expression	Allows export of unspliced and partially spliced transcripts from nucleus
vif	Viral infectivity	Affects particle infectivity
vpr	Viral protein R	Transport of DNA to nucleus. Augments virion production. Cell cycle arrest
vpu	Viral protein U	Promotes intracellular degradation of CD4 and enhances release of virus from cell membrane
nef	Negative-regulation factor	Augments viral replication *in vivo* and *in vitro*. Downregulates CD4 and MHC class II

Figure 1.4: The genes of HIV-1 (Janeway, Jr., 2001)

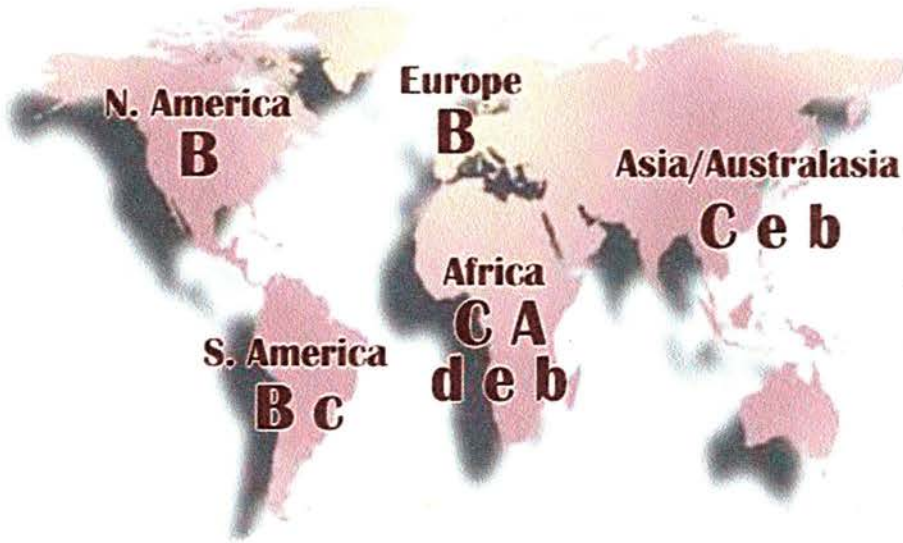


Figure 1.5: World map showing distribution of HIV-1 group M sub-types. Dominant subtypes is each area are shown by capital letters. nb E/e represent CRFA_E (Hunt 1999).

Europe the impact of the virus has been far less than in many other areas and most infections have been with subtype B.

Some developing countries initially had a limited subtype B epidemic before another subtype emerged to cause higher levels of infection (Weniger et al., 1994; Subbarao et al., 1998; Janssens et al., 1997). In addition, mode of transmission is strongly correlated with subtype. For example subtype C is usually transmitted by heterosexual sex while subtype B is associated with intravenous drug use and homosexual sex (Essex, 1999). These phenomena have led some to suggest that certain subtypes may differ biologically. A disproportionate amount of research has focused on subtype B due to its prevalence in the richer countries of Europe and the Americas and it is now important to establish if the findings can be applied to all subtypes.

1.4 Life Cycle

The retroviruses have a unique and fascinating life cycle which enables them to insert their genome into the host cell genome. Thus the host cell machinery becomes subverted to produce viral proteins. The main stages of the life cycle are outlined in figure 1.6 and further described below. A more detailed description of the specific roles played by each of the viral proteins is given in sections 1.5-1.7.

1.4.1 Adsorption and fusion

The first stage in the life cycle is adsorption and fusion (figure 1.7). HIV attaches to the cell by an interaction between the gp120 portion of Env and host cell CD4. It appears that gp120 must interact with three different CD4 molecules to allow fusion to proceed (Pierson and Doms, 2003). This

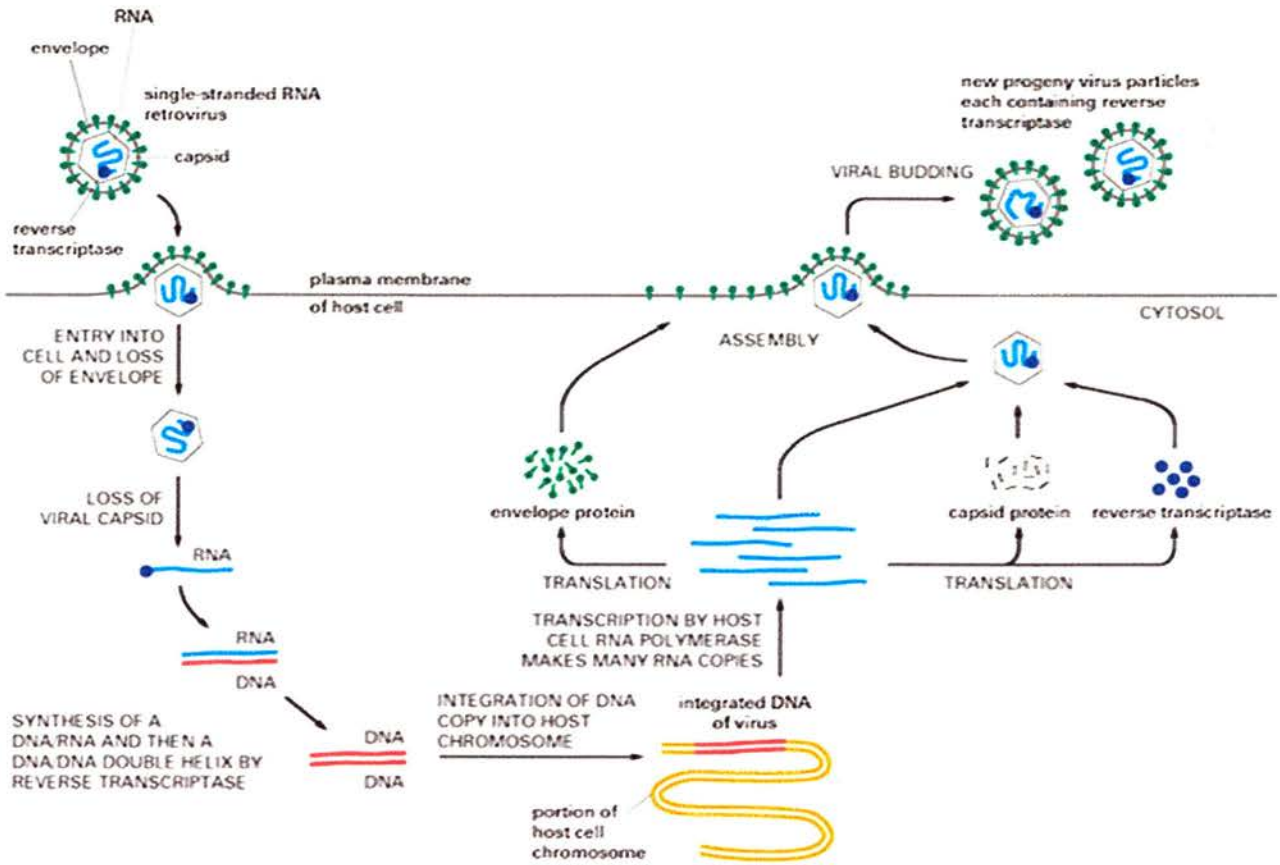


Figure: 1.6: Diagramatic representation of HIV life cycle. The virion fuses with the host membrane and releases its contents into the cell. RNA is reverse transcribed into single stranded DNA. Double strand synthesis takes place. The double stranded DNA genome inserts into the host genome. RNA is transcribed and translated. Newly synthesised viral peptides along with newly transcribed genomic RNA accumulate at the host membrane and bud out forming new virions (Alberts et al., 1998).

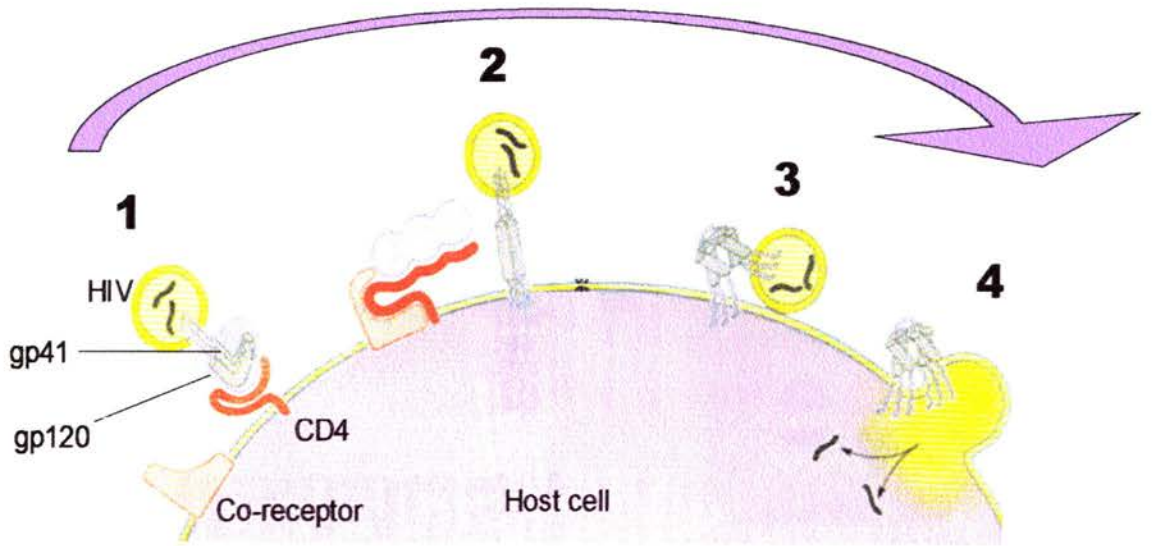


Figure 1.7: Attachment and membrane fusion of HIV with host cell
1: gp120 interacts with CD4 causing a conformational change in gp120; 2: gp120 binds to co-receptor causing a conformational change in gp41 which allows it to insert into the host membrane; 3: fusion of viral and host membranes and 4: release of viral contents into cell (Adapted from Hunt 1999).

interaction causes a conformational change in gp120 revealing a site which binds to a co-receptor. *In vivo* the co-receptor is usually either CCR5 or CXCR4 (see section 1.11). Co-receptor binding causes a further conformational change which enables the gp41 portion of Env to insert into and fuse with the host cell membrane releasing the contents of the virion into the cell.

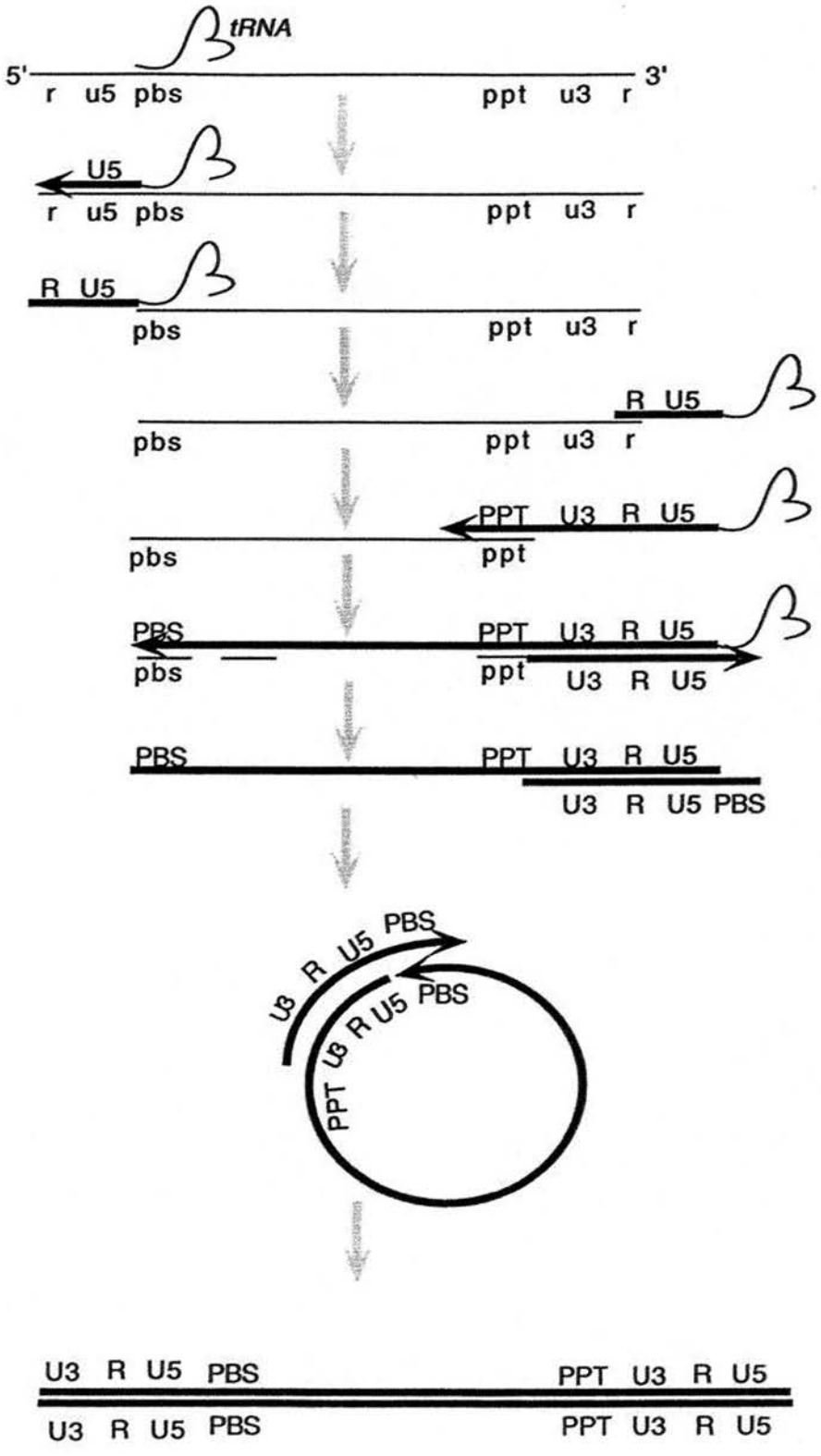
1.4.2 Uncoating

After the contents of the virion have entered the cell, components which do not form part of the pre-integration complex (PIC) must be shed. This process is known as uncoating (reviewed in Dvorin and Malim, 2003). Three virion components have been shown to play a role in uncoating. These are Nef, Vif and the host derived protein, cyclophilin A. Defects in, or lack of these molecules has been shown to reduce the efficiency of a step after viral entry and before completion of reverse transcription (Piguet and Trono, 1999; Vonschwedler et al., 1993; Braaten et al., 1996). The mechanism by which any of these molecules enhance viral uncoating remains unclear.

1.4.3 Reverse Transcription

Reverse transcription of the viral RNA genome into double stranded DNA is an early step of the life cycle (reviewed in Goff, 2001) (figure 1.8). The reaction occurs in a complex known as the pre-integration complex (PIC) containing MA, NC, Vpr, RT, IN, viral RNA and a tRNA molecule incorporated from the host cell from which the virion budded.

Figure 1.8: Reverse transcription of HIV genome. Thick lines represent DNA and thin lines represent RNA. The tRNA molecule binds to the primer binding site (pbs) near the 5' end of the RNA genome. DNA synthesis, catalysed by RT, occurs in a 5' to 3' direction forming the so called minus-strand strong-stop DNA. This short piece of single stranded DNA jumps to the 3' end of the RNA genome. In order to do this, the portion of RNA which has already been copied must by degraded. This is achieved by the RNase H activity of RT. The strong-stop DNA is now able to move to the 3' end where it re-aneals to the repeat region (r). RT continues to catalyse DNA synthesis in a 5' direction with the copied RNA being deleted by RNase H as the synthesis machinery moves along. Two portions of the RNA template known as the ppts are resistant to RNase H degradation and remain annealed to the DNA. These can then act as primers for plus-strand DNA synthesis which continues past the end of the genome to include the first 19 bases of the tRNA. This piece of DNA is refered to as the plus-strand strong-stop DNA. The tRNA molecule is degraded by RNase H leaving the plus-strand strong-stop DNA with an unpaired PBS. This pairs with the 3' end of the minus strand DNA creating a circular form. DNA synthesis occurs from the 3' end of both strands. The already formed U3, R and U5 regions of the strands peel away and act as templates and thus a linear piece of DNA is formed with the characteristic long terminal repeats at each end. (Goff 2001)


tRNA
5'
3'
r u5 pbs
ppt u3 r
U5
R U5
PPT U3 R U5
PBS
U3 R U5 PBS
PPT U3 R U5

1.4.4 Nuclear Import

The ability of HIV to infect non-dividing cells relies on the PIC being able to travel to the nuclear membrane and enter the nucleus (reviewed in Strebel and Bour, 1999; Dvorin and Malim, 2003). The mechanism by which the PIC travels to the nuclear membrane is unclear. A number of other viruses, including herpes simplex virus 1 and adenovirus, travel along components of the host cell cytoskeleton in order to reach the nucleus (Sodeik et al., 1997; Leopold et al., 2000). The cytoskeleton forms the interior framework of cells and consists of intermediate filaments, actin and microtubules (reviewed in Dvorin and Malim, 2003). Molecules can be transported in either direction along the latter two components by association with motor proteins. Along actin filaments these are the myosin family for plus end transport and myosin VI for minus end transport. Along microtubules, the kinesin family mediates plus end transport while the dynein family, move in a minus end direction. There has been some evidence that interactions between the PIC and actin filaments are important in an early stage of PIC transport (Bukrinskaya et al., 1998; Iyengar et al., 1998). However, recent studies have implicated microtubules as the main mediator of PIC transport to the nucleus. Using fluorescent labelling, PICs were visualized moving along microtubules (Dvorin and Malim, 2003). Further research is required to fully characterize the mechanism of PIC transport.

Once at the nuclear membrane, the PIC uses the host's nuclear import machinery to enter the nucleus. The intact PIC is estimated to have a diameter of around 56nm. This is too large for normal signal mediated import

through the nuclear pore suggesting that either a conformational change or shedding of some components must occur before entry (Miller et al., 1997; Pante and Kann, 2002). MA and INT possess putative nuclear localization signals (NLS) which may bind to the dimer of importin α and importin β (Gallay et al., 1997; Gallay et al., 1996). Vpr also localizes to the nucleus and is able to bind both importin α and the nuclear pore itself (Popov et al., 1998; Fouchier et al., 1998). It has been suggested that Vpr plays a role in stabilizing the interaction of MA and INT with the importin proteins (Popov et al, 1998). Despite these findings, nuclear import can occur in the absence of both MA and Vpr and a transferable NLS has not been identified on INT (Dvorin and Malim, 2003). Controversially, some have suggested that import of the DNA provirus can occur in the absence of any viral proteins via interactions with host cell DNA-binding nuclear import proteins (Dvorin et al., 2002).

1.4.5 Integration

Once in the nucleus the provirus must be integrated into the host genome before the cell will efficiently produce progeny virus (Sakai et al., 1993). Integration is catalysed by viral INT (reviewed in Goff, 2001; Engelman, 2003) (figure 1.9).

Purified PICs are unable to mediate integration alone however the addition of cellular extracts restores this activity (Farnet and Bushman, 1997). This suggests that host factors are required for efficient integration. Two host DNA binding proteins, BAF and HMAGA1, which form stable nucleoprotein complexes, have been identified (Engelman, 2003). The formation of such

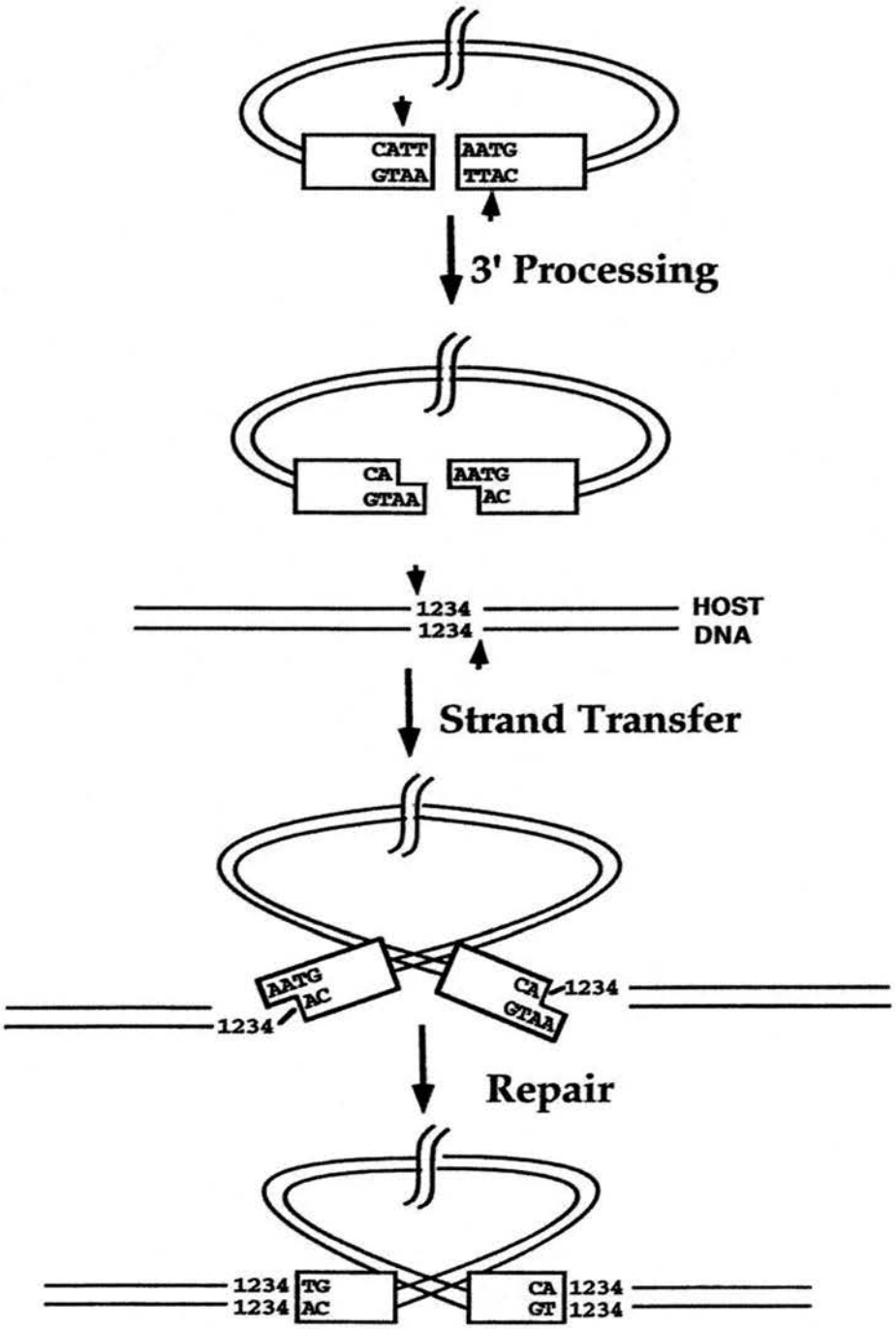


Figure 1.9: Integration of provirus into host genome. The first step, known as 3'-end processing, involves the removal of two nucleotides from the 3' end of each DNA strand. This results in a "sticky" ended piece of linear DNA with recessed 3'-ends and protruding 5'-ends. In the second step, known as strand transfer, the 3'-end of the viral provirus attacks the phosphodiester bond of the target DNA at the site where integration will occur. INT catalyses the binding of the 3'end to the host DNA. (Goff 2001)

complexes is thought to inhibit intramolecular integration; i.e. integration of the end of the provirus into another part of the proviral sequence. It is thought that BAF may have the additional function of binding host cell DNA to allow intermolecular integration.

The repair of the nicked DNA and binding of the 5'end to the host DNA is thought to be mediated by both viral and host enzymes (Engelman, 2003). The steps involved are thought to be DNA synthesis to fill in single strand gap, cleavage to remove viral 5' dinucleotide overhang and ligation of cleaved 5' end to newly synthesised DNA.

1.4.6 Transcription and Translation of Viral Genome

Transcription of HIV genes is catalysed by the host polymerase RNA II (reviewed in Freed and Martin, 2001). The loading of this enzyme onto the viral genome relies on promoter elements located in the viral LTR and host cell transcription factors (TF). The HIV core promoter, which is necessary for basal levels of RNA synthesis, consists of the TATA element and three Sp1 binding sites. TFIID first binds to the TATA region located upstream of the transcription start site. TFIID binds TFIIB which mediates Pol II recruitment to promoter area. Pol II must be phosphorylated in order to produce full length transcripts. A number of cellular kinases are able to achieve this. Tat plays a role in recruiting one such kinase and thus promoting transcription (see section 1.6). Sp1 is a constitutively expressed host transcription factor. Binding of Sp1 to at least one of its viral binding sites markedly increases levels of transcription.

The two NFκB binding sites upstream of the core promoter provide cellular

activation dependent enhancement of viral transcription. In quiescent T cells NFκB is found only in the cytoplasm. Upon activation, NFκB rapidly accumulates in the nucleus. Its physiological role is to increase transcription of various cellular genes following activation but in HIV infected cells can also increase transcription of HIV (Alcami et al., 1995).

RNA splicing is a vital feature of HIV gene expression (reviewed in Goff, 2001; Alonso and Peterlin, 1999). When HIV is first transcribed, all the RNA transcripts are multiply spliced before export to the cytoplasm so that only the regulatory proteins Tat and Rev are expressed. As the concentration of these proteins build up they facilitate increased transcription and export of unspliced and partially spliced RNA transcripts (see section 1.6). These act as templates for production of other viral proteins.

Unspliced RNA acts as a template for Gag and Pol production. In most cases translation results in production of Gag only as Pol lies in a different reading frame. However, approximately 10% of the time, the ribosome "jumps back" a nucleotide due to a RNA slippy sequence. This puts the ribosome in the correct frame for *pol* translation and thus a Gag/Pol fusion protein is formed. Singly spliced RNA, in which most of the *gag* and *pol* sequences have been removed, is used for the translation of *env* while other viral genes are translated from multiply spliced transcripts (reviewed in Freed and Martin, 2001). Most viral genes are translated by cytoplasmic ribosomes however *env* is translated in endoplasmic reticulum (ER) associated ribosomes (see section 1.5.3).

1.4.7 Assembly and Budding

The final step in HIV's life cycle is for all the newly formed viral components to assemble at the host membrane and bud out as a immature virus particle (reviewed in Greene and Peterlin, 2002; Gottlinger, 2001). The assembly of viral proteins at the host cell membrane is mainly mediated by the Gag polyprotein. In fact Gag can direct the formation of virus like particles even in the absence of other viral genes. Approximately 2000 Gag proteins are thought to be required to make just one HIV virion (Wilk et al., 2001). The precise roles of the various domains of Gag are discussed in more detail in section 1.5.1. Once the necessary components are assembled at the membrane the virion is able to "bud" out coating itself in the host membrane. It is thought that the virus achieves this by subverting the host cell's endocytotic mechanism for production of multi-vesicular bodies.

1.5 Structural Proteins

1.5.1 Gag

The Gag proteins play an important role in directing the assembly of HIV particles (reviewed in Gottlinger, 2001). Gag is initially translated as a polyprotein and in this form it mediates the formation of immature viral particles. To become infectious, the virion must undergo a maturation step in which the Gag polyprotein is cleaved by viral protease into its constituent parts.

1.5.1.1 Matrix

The matrix (MA) domain consists of a N-terminal globular "head" formed by four alpha-helixes and a C-terminal alpha-helical "tail" (reviewed in Turner

and Summers, 1999). MA is involved in targeting the Gag polyprotein to the plasma membrane (reviewed in Gottlinger, 2001). Essential to this function is a myristic acid moiety which covalently attaches to an N-terminal glycine in MA (Gottlinger et al., 1989). The globular head is also thought to be important in the affinity of MA for membranes. This part of the domain forms trimers which have conserved basic residues on their outer surface. These may interact with the acidic phospholipids heads found in membranes. Indeed mutations to these basic domains disrupts virus assembly (Zhou et al., 1994).

It is thought that binding of the myristic acid to membranes is initially prevented by the conformation of MA and a conformational change is required in order to reveal the acid moiety (Spearman et al., 1997). This mechanism, known as a "myristic switch", would explain the finding that mutations to (Paillart and Gottlinger, 1999) or even complete deletions of (Reil et al., 1998) the globular head of MA can lead to dramatic increases in virus particle production; these disruptions may expose the myristic acid and thus increase affinity for membranes. MA also plays a role in the incorporation of Env into budding virions (Dorfman et al., 1994).

1.5.1.2 Capsid

Capsid (CA) consists of two largely alpha-helical domains separated by a flexible linker sequence (reviewed in Turner and Summers, 1999).

The N-terminal domain of CA binds the enzyme cyclophilin A (a peptidyl-prolyl cistrans isomerase) and mediates its incorporation into HIV virions (Luban et al., 1993). The presence of this enzyme in virions increases

infectivity; however the precise mechanism by which it achieves this remains unclear (Gamble et al., 1996).

The first 20 amino acids of the C-terminal domain of CA constitute the Major Homology Region (MHR) which is conserved in all retroviruses (Gamble et al., 1997). The remainder of the domain along with a portion of the adjacent p2 "spacer" peptide mediate Gag dimerisation *in vitro* and are thought to play a role in correct virion assembly *in vivo*. Cleavage between the C-terminal domain of CA and p2 during virus maturation is thought to lead to the formation of the characteristic cone shaped capsid (Accola et al., 1998).

1.5.1.3 Nucleocapsid

The major function of the nucleocapsid (NC) domain is the specific encapsulation of genomic HIV RNA into developing virions (Berkowitz et al., 1993). Crucial to this function are two zinc finger domains joined by a highly basic linker domain (Bess, Jr. et al., 1992; Summers et al., 1992). The basic linker domain results in non-specific binding to RNA while the zinc-fingers bind specifically to the Ψ-site.

A second function is the packaging of Gag into virions. The non-specific, RNA binding activity of the basic linker sequence is crucial to this. Binding of multiple NCs to RNA is thought to promote dimer formation (Zhang et al., 1998). This appears to be an important first step in virion assembly. Interactions between NC and cytoskeletal components may facilitate Gag transport (Rey et al., 1996).

NC also plays a role in thermodynamic folding of the RNA genome, RNA dimerisation, tRNA binding and strand transfer (Rein et al., 1998).

1.5.1.4 p6

p6 protein plays a role in virion release following assembly at the host membrane (Gottlinger et al., 1991). It appears that p6 acts together with PR and host cell ubiquitin to mediate virion release (Schubert et al., 2000). The incorporation of Vpr into virions is also mediated by p6 protein (Paxton et al., 1993).

1.5.2 Pol

1.5.2.1 Protease

HIV protease (PR) exists as dimers and a cleft between the two monomers functions as the enzyme's active site (reviewed in Turner and Summers, 1999). PR functions to cleave a number of viral polypeptides (reviewed in Freed and Martin, 2001). The precise amino acid signal which triggers the enzyme to cleave a target is unclear; however a number of sites are known to be cleaved with various efficiencies. The first cleavage event releases PR itself. It is unknown if this step is self-catalytic or if another molecule of PR performs this function. PR goes on to liberate the various domains of Gag and Pol. Nef is also cleaved although the reason for this is not known (Bukovsky et al., 1997). PR becomes most active as the virions are budding from the membrane (Kaplan et al., 1994). The release of the various Gag domains at this time results in the dramatic alteration in morphology known as virion maturation.

1.5.2.2 Integrase

HIV Integrase (INT) has three catalytic activities: 3'-end processing, cleavage of host DNA and ligating the 3'-end viral strands to 5'-end host strands (strand transfer). These activities allow INT to catalyse the integration of the

DNA provirus into the host cell genome as outlined in section 1.4.5

INT has three structural and functional domains (reviewed in Turner and Summers, 1999). The N-terminal zinc-finger containing domain contributes to enzyme stability via multimer formation. The core region has a secondary structure analogous to other polynucleotidyl transferases and is the main catalytic part of the enzyme. It is able to bind DNA only in the presence of a divalent metal ion. The C-terminal domain is required, along with the core for 3'-end processing and integration. It shows non-specific DNA binding which may be important in maintaining INT's association with nucleic acid during reverse transcription.

1.5.2.3 Reverse Transcriptase

HIV Reverse Transcriptase (RT) has three enzymatic activities (reviewed in Goff, 2001): RNA-templated DNA synthesis, RNaseH activity for degradation of tRNA and RNA base-paired with DNA and DNA-templated DNA synthesis. Together these actions allow HIV to convert its RNA genome into double stranded DNA which can integrate into the host genome (section 1.4.3).

RT is a heterodimer of p66 and p55 peptides (reviewed in Turner and Summers, 1999). The p55 peptide is produced by PR cleavage of p66 to release the RNAseH domain. Surprisingly, the secondary folding of the two monomers differs dramatically. The structure of p66 has been compared to a partially open hand with the active site lying in the palm between the thumb and finger domains. This active site contains three aspartic acid residues which are essential for the enzyme's function. In p55 these residues are buried within the peptide suggesting that it does not have an enzymatic role.

The high mutation rate of RT is critical in influencing viral evolution and this is discussed further in section 1.9.

1.5.3 Env

The 160kD Env precursor protein is synthesised in ER associated ribosomes. A number of modifications to the protein occur in the ER. It becomes heavily glycosylated such that half of its mass consists of oligosaccharide chains which are further modified during Env's journey to the cytoplasmic membrane (Leonard et al., 1990). These are thought to play a role in inhibiting antibody binding to the mature protein (Profy et al., 1990). Trimerisation, disulphide bond formation and association with a host chaperone molecule also occur within the ER. Env is then transported to the Golgi body. Within this compartment cleavage by cellular furin occurs separating the surface gp120 from the transmembrane gp41. From the Golgi body Env is transported to the cytoplasmic membrane. The two subunits remain attached to each other by weak non-covalent interactions which allow a large amount of gp120 to be shed from the cell surface (Freed and Martin, 2001).

1.5.3 gp41

The structure of various portions of HIV gp41 has been solved (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997). In addition the structure of the almost intact gp41 of SIV has been elucidated (Caffrey et al., 1998) and it is assumed that the HIV gp41 structure closely mirrors this. Each monomer of gp41 has a rod-like structure formed by two anti-parallel α-helices. Protruding from one end of the rod is an extended loop which is able to bind gp120. From the other end emerges both the C-terminal end of the protein,

which passes through the viral membrane and interacts with MA, and the N-terminal end which contains the fusogenic peptides (Caffrey et al., 1998).

The mechanism for viral/host fusion is thought to be a "spring-loaded" action (reviewed in Turner and Summers, 1999). In this model there would be a conformational change in gp41, triggered by binding of a co-receptor to gp120. This would result in the two α-helical portions, which are normally folded back on each other, become extended to form one long helical rod with the fusogenic peptide exposed at the end. Following insertion of the fusion peptide, gp41 would fold back into the original conformation thus bringing the viral host membranes into close proximity (Melikyan et al., 2000). It has been suggested that the energy released upon refolding to the more stable structure may aid membrane fusion. This mechanism mirrors that found in the envelope protein, haemagglutinin (HA), of the influenza virus (Carr and Kim, 1993). Indeed there are structural similarities between HIV gp41 and HA in both primary and secondary structural levels.

1.5.3.2 gp120

The details of the secondary structure of gp120 are complex, involving numerous overlapping and intersecting β-sheets and α-helices. Essentially it forms two globular masses separated by a β-sheet (reviewed in Turner and Summers, 1999). There are 5 conserved (C1-C5) and 5 variable (V1-V5) regions (Starcich et al., 1986). Four of the variable regions (V1-V4) form loops extending from the surface with disulphide bonds at their base.

CD4 binds in the oligosaccharide-free cleft between the two globular parts. This interaction forms a hydrophobic pocket formed by the deep cleft and

covered by CD4. Surprisingly, many of the residues lining this cavity are highly conserved although they do not interact directly with CD4 (Kwong et al., 1998). A larger cavity is also formed around CD4. In this case the non-interacting residues are not conserved however they are surrounded by conserved CD4 binding sites. This highly unusual structure is thought to contribute to the difficulty in raising antibodies to the CD4 binding site (reviewed in Poignard et al., 2001). Binding of CD4 induces a conformational change which exposes the chemokine receptor binding site. It is unclear if this conformational change involves just a change of position of the variable loops or if a much more fundamental conformational change occurs after CD4 binding (Kwong et al, 1998).

1.6 Regulatory Proteins

The first viral proteins to be produced are the transactivator of transcription (tat) and regulator of viral expression (rev). These proteins regulate and amplify the production of other viral products (reviewed in Freed and Martin, 2001; Alonso and Peterlin, 1999).

1.6.1Tat

Tat is encoded by two exons. These encode the three domains which contribute to Tat's transactivation activity: the activation and basic domains (see later) and the C-terminal domain which may have a minor auxiliary role in transactivation (Sadaie et al., 1988).

The function of the activation domain is to recruit host factors which in turn act to enhance transcription. The critical host factors for this function are the heterodimer of CDK9 and cyclin T1. CDK9 is a kinase which is able to

hyperphosphorylate the CTD of Pol II (Marshall et al., 1996). This alters the enzymatic activity of Pol II from initiation of transcription to transcriptional elongator. The cyclin T1 portion of the heterodimer binds Tat and also enhances the Tat/TAR interaction (Wei et al., 1998).

The basic domain of Tat contains an arginine rich motif which has two functions. Firstly, it allows Tat to bind to its RNA target, the transactivation response region, or TAR (Dingwall et al., 1989). TAR is found at the five prime end of both spliced and unspliced HIV viral transcripts. It consists of a base paired stem, containing a trinucleotide unpaired "bulge", with a hexanucleotide Guanine (G) rich loop at the end (Feng and Holland, 1988). Tat interacts mainly with the bulge region while cyclin T interacts mainly with the loop region. In addition this domain acts as a nuclear localization signal (Hauber et al., 1989).

1.6.2 Rev

HIV uses the host cell machinery for post translational modifications of RNA such as capping, 3'-end cleavage, polyadenalation and splicing (reviewed in Freed and Martin, 2001). The default pathway for transcripts involves the complete splicing out of all introns prior to nuclear export. This mechanism produces the multiply spliced mRNAs which encode Tat, Rev and Nef. Other transcripts are not exported by this pathway due to the presence of cis-acting AU-rich regions which cause nuclear retention. In order to produce other viral proteins, unspliced or partially spliced transcripts must be exported by an alternative means and this is achieved by Rev (Regulator of Viral Expression).

Like Tat, Rev is encoded by two exons however in the case of Rev both

exons are required for its primary function. Rev has two functional domains: an arginine-rich domain which includes a nuclear localization signal (NLS) and a hydrophobic region containing a nuclear export signal (NES) (reviewed in Strebel and Bour, 1999).

Rev exports transcripts via a pathway involving interactions with numerous cellular factors. Critical to the process is the GTPase, Ran. This exists mainly as Ran-GTP in the nucleus while in the cytoplasm it is mainly in the form of Ran-GDP (Izaurralde et al., 1997). Another essential host factor is the nuclear pore complex (NPC). This is a multimer of up to one hundred different proteins known as nucleoporins (Fabre and Hurt, 1997).

Rev is produced in the cytoplasm from the early mRNA transcripts. It then binds directly via its NLS to Importin-β which directs its import through the NPC. The binding of Ran-GTP to Rev allows it to dissociate from Importin-β and bind to the cis-acting Rev Responsive Element (RRE). The RRE is a highly folded cis-acting region found in unspliced and partially spliced HIV transcripts. Multimerisation of Rev occurs around the RRE masking the NLS while exposing the NES. To facilitate export, Rev then binds via its NES element to the exportin peptide CRM1. Ran-GTP is required to stabilize this interaction. CRM1 is able to interact with nucleoporins to mediate the export to the cytoplasm of the entire complex of Rev, RNA transcript, Ran-GTP and CRM1. In the cytoplasm, Ran-GTP is hydrolysed to Ran-GDP and this allows the dissociation of the exported components (Strebel and Bour, 1999).

1.7 Accessory Proteins

HIV possesses four accessory proteins which have various roles in viral

pathogenesis (reviewed in Steffens and Hope, 2001; Strebel and Bour, 1999).

1.7.1 Nef

The 27 kDa Nef protein is the first viral protein to accumulate following HIV infection of a cell. It is thought to play at least three distinct roles in HIV pathogenesis (reviewed in Strebel and Bour, 1999).

Nef contributes to the cellular activation of T-cells. The gene expression profiles after either Nef mediated or normal T-cell receptor mediated activation are remarkably similar and certain T-cell receptor components are required for Nef activation (Simmons et al., 2001). These findings suggest that Nef may activate cells by mimicking T-cell receptor signalling. A Nef associated cellular kinase, p21-associated kinase 2 (PAK2), has been identified as a potential mediator of Nef-induced cellular activation (Arora et al., 2000).

Nef expression can also result in down-regulation of certain host cell surface molecules by either clatherin-mediated or non-clatherin dependent endocytotic pathways (Swigut et al., 2001; Luo et al., 2001). Packaging of Nef into virions increases viral infectivity by an unknown mechanism (Miller et al., 1994).

1.7.2 Vpr

Vpr is incorporated into viral particles via an interaction with p6 at a rate of between 100-200 copies per virion (Muller et al., 2000). Like Nef, it appears to have numerous activities which are to some degree genetically distinct (reviewed in Steffens and Hope, 2001; Zhu et al., 2001).

Vpr causes infected cells to arrest in the G2 phase of the cell cycle by inhibition of the cyclinB/p34cdc2 complex which is crucial in initiation of mitosis (Re et al., 1995; He et al., 1995). Along with MA and INT, Vpr plays a role in facilitating nuclear import of the pre-integration complex. MA and INT associate with the Importin-α/Importin-β heterodimer via their NLS sequences. It appears that Vpr may stabilize this interaction allowing the entire complex to be imported (Popov et al, 1998).

1.7.3 Vpu

Vpu forms multimers which lodge in the membranes of infected cells. It has two known functions (reviewed in Steffens and Hope, 2001; Strebel and Bour, 1999). Firstly it binds CD4 and facilitates its degradation. It achieves this by binding the host protein h-TrCP which activates Skp1 which in turn degrades CD4 (Margottin et al., 1998). Additionally Vpu plays a role in virion release although the mechanism of this remains unclear (Marassi et al., 1999).

1.7.4 Vif

The *vif* gene encodes a 23 kDa basic protein which is crucial for viral infectivity (Fisher et al., 1987). Its cellular target has recently been identified as the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (Sheehy et al., 2002). In the absence of Vif, this cellular protein is incorporated into virions and converts cytosine residues to uracil residues in newly synthesised viral DNA (Zhang et al., 2003). It is thought that this action may destabilise the DNA and thus lead to its degradation by host cell enzymes. Vif blocks the incorporation of APOBEC3G into virions and thus leads to an increase in viral infectivity (Marin et al., 2003).

1.8 Combating HIV

1.8.1 Host Immunity

The interplay between HIV and the host immune system is highly complicated. As outlined previously (section 1.2), HIV can have a devastating effect on many aspects of host immunity. Indeed certain arms of the immune system, such as some complement responses, appear to actively promote HIV pathogenesis. On the other hand, some host responses are useful in combating HIV. The study of long term non-progressors (LTNP), HIV-infected individuals who remain disease free for ten years or more, has been particularly useful in identifying responses which prevent or slow down pathogenesis. In addition, the immune responses found in multiply exposed non-infected (MENI) individuals have been studied in the hope of identifying responses which could protect against initial infection.

1.8.1.1 Innate responses

The innate immune response consists of a plethora of interacting cellular and soluble factors. The interactions between innate responses and HIV have recently attracted increased attention and a number of factors which appear to correlate with protection against disease have been identified.

Mannose binding lectin (MBL), a circulatory protein, is able to bind and thus opsonise HIV virions or target them for destruction by complements components (Ezekowitz et al., 1989; Saifuddin et al., 2000). Low levels of MBL or polymorphisms in the MBL receptor are associated with increased risk of HIV infection, faster progression to disease and shorter survival periods post-AIDS diagnosis (Pastinen et al., 1998; Boniotto et al., 2000).

The ability to produce type 1 interferon (IFN) in the face of pathogen challenge is associated with long term survival of HIV infected individuals (Lopez et al., 1983). The major cellular producers of IFN are a small population of peripheral leukocytes known as plasmacytoid dendritic cells (PDCs) (Siegal et al., 1999). These cells have a formidable ability to secrete IFN following exposure to HIV-infected T cells or, to a lesser extent, free virions (Levy et al., 2003). It appears that high numbers of PDCs may be able to delay disease onset even in the face of low CD4 T cell numbers (Soumelis et al., 2001).

CD8 T cells are also capable of generating protective innate responses to HIV. These cells are better known for their adaptive cytotoxic function however, they are also able to secrete a CD8 anti-HIV factor (CAF) which inhibits a wide variety of HIV-1, HIV-2 and SIV variants (Levy et al., 1996). This factor remains to be fully characterised but appears to involve a protease activity (Mackewicz et al., 2003). The ability of CD8 cells to mount this response is associated with long term survival of HIV infected individuals (Barker et al., 1998).

Natural killer (NK) cell function may be correlated with reduced susceptibility to HIV infection and slower disease progression (Nagachinta, et al., 1997; Ratcliffe, et al., 1994). Ex vivo studies have shown that NK cells can exert a suppressive effect on HIV infected CD4 T cells both by release of soluble CC-chemokines and by cell to cell contact (Kottilil, et al., 2003). Both these effects are inhibited by high levels of viraemia (Kottilil, et al., 2003; Eger & Unutmaz, 2004).

1.8.1.2 Adaptive responses

The emergence of cytotoxic CD8 T lymphocytes (CTLs) is thought to be the major factor in the dramatic reduction of viral loads seen following primary infection (Koup et al, 1994) and throughout infection levels of CTLs are inversely correlated with plasma viral loads (Ogg et al., 1998). If this response is not sustained, patients progress rapidly to AIDS (Hay et al., 1999). Despite this, CTLs do not seem able to completely clear HIV and thus the virus persists and eventually causes AIDS. LTNP and MENI individuals have been extensively studied in an attempt to characterise particularly effective CTL responses. It appears that CTL responses with broader specificities may be related to longer survival (Harrer et al., 1996). It is unclear whether the breadth of the response, or the presence within this response of CTLs specific for certain antigens underlies the slow disease progression. One theory is that the emergence of CTL responses to early HIV proteins such as Tat and Rev is responsible for protection (Vanbaalen et al., 1997).

1.8.2 Vaccination

Despite a huge amount of research there is still no effective vaccine against HIV. A major difficulty in developing a vaccine which protects against HIV infection is that it is not clear what immune responses such a vaccine would need to stimulate. It is hoped that the study of MEDI individuals might lead to better characterisation of protective responses. Perhaps a more achievable aim would be the production of a vaccine which delayed or even prevented the progression to AIDS in HIV infected individuals. There has been extensive research of LTNP in an attempt to define responses which delay

disease. Alternatively, immune responses which are not normally seen in the course of HIV infection might prove protective against infection or disease if stimulated artificially.

Initial research into anti-HIV vaccines attempted to elicit an antibody response to HIV Env. The results of these trials were largely disappointing as the antibody produced was not capable of neutralising heterologous HIV strains (Moore et al., 1995). However, more recently a number of antibodies have been identified which appear to have broad specificity for a range of HIV variants (Moore et al., 2001). Passive immunisation with these neutralising antibodies (nAbs) is protective in monkeys against a challenge with SIV/HIV (SHIV) chimeric virus (Mascola et al., 2000). Researchers are now investigating potential vaccine strategies which would stimulate the production of these nAbs *in vivo*.

Following disappointing early results with antibody-stimulating vaccines, most recent research into potential anti-HIV vaccines has concentrated on eliciting CTL responses. Although these would not be able to prevent initial infection they may be able to delay the onset of disease. In order to stimulate CTL responses, HIV antigens must be presented by host dendritic cells (DCs). Vaccine trials in animal models have been carried out using a variety of constructs designed to deliver antigenic peptides to DCs. These have included naked plasmid DNA encoding HIV peptides (Amara et al., 2001); recombinant viruses encoding HIV/SIV peptides (Zhong et al., 2000; Engelmayer et al., 2001); liposomes containing HIV peptides (Zheng et al., 1999) or HIV peptides linked to a lipid tail (Andrieu et al., 2000). The results

of these trials have been promising however there are still many obstacles to overcome. The magnitude of CTL response generated needs to be increased; perhaps by using adjuvants or novel prime/boost combinations. In addition, strategies to maintain T cell responses over time need to be further investigated.

1.8.3 Drug treatment

The first antiretroviral agent to be licensed was the RT inhibitor zidovudine in 1987. Initial trials of on individuals with AIDS or ARC had shown that there was a significant decrease in mortality when zidovudine was taken compared to the placebo control (Fischl et al., 1987). Unfortunately the clinical benefits of taking zidovudine alone decrease dramatically over time mainly due to the rapid emergence of resistant variants (Volberding et al., 1994). Since the discovery of zidovudine numerous other antiretroviral agents have been identified and licensed. These have all been inhibitors of either RT or PR. Taking a combination of drugs with distinct targets results in a significant delay in the emergence of drug resistant variants. In particular, since the emergence of PR inhibitors in the mid-nineties, HIV infected individuals can be treated with a combinations of drugs known as highly active anti-retroviral treatment (HAART). Such regimes are able to suppress viral replication below the level of detection for many years although they do not eradicate the virus from cells (Menendez-Arias, 2002; Weller, 1999).

Anti-retroviral agents produce a number of adverse side effects of varying severity (Weller, 1999). Zidovudine can cause haematological disturbances such as anaemia and neutropenia. Neuropathies can occur with didanosine

or zalcitabine treatment. Both nevirapine and efavirenz can cause skin rashes. Most of the inhibitors of RT can also cause gastrointestinal disturbances in some patients. Perhaps the most unacceptable side effect for patients, however, are the disturbances of lipid metabolism associated with PR inhibitor use. These can lead to abnormal fat distribution: sub-cutaneous fat is lost from the face, limbs and upper body while increased fat deposits accumulate around the abdomen, breasts and upper back.

1.9 Viral Evolution

HIV has a remarkably high rate of evolution. A number of viral and host factors have an influence on this.

1.9.1 Viral factors

The HIV genome is copied many times during the HIV life cycle: RNA templated minus strand DNA synthesis catalysed by viral RT; DNA-templated plus-strand DNA synthesis catalysed by viral RT; DNA-templated DNA replication catalysed by cellular DNA polymerases and DNA templated genomic RNA synthesis catalysed by cellular RNA polymerase II. The *in vivo* point mutation rate of HIV has been estimated as 3.4×10^{-5} (Mansky and Temin, 1995). This rate is relatively high compared with cellular genes suggesting that one or both of the polymerases unique to HIV replication (i.e. viral RT and RNA polymerase II) have a higher mutation rate than host polymerases. An additional mechanism of viral diversity is recombination.

1.9.1.1 Reverse Transcription

RT contributes to viral evolution due to its relatively low fidelity (reviewed in McGrath et al., 2001; Preston and Dougherty, 1996). Unlike cellular

polymerases, RT lacks a 3' to 5' exonuclease which could correct any mis-incorporations. The *in vivo* rate of RT is around 20 times lower than the estimated mutation rate of purified HIV RT suggesting that *in vivo* factors act to modulate RT fidelity. One such factor could be the presence of Vpr. It binds the host DNA repair enzyme, uracil DNA glycosylase and facilitates its incorporation into virions (Mansky et al., 2000).

1.9.1.2 RNA polymerase II

Once integrated, the DNA provirus is copied into RNA by host cell RNA polymerase II. In the host cell, this enzyme does not contribute to genomic mutation rates as the RNA is used only as a template for protein synthesis. For this reason, there is not a strong selection pressure on this enzyme to maintain a high fidelity. Thus, in common with HIV RT, this enzyme lacks a proof-reading mechanism. In the case of HIV however, the RNA produced by this enzyme is used both to translate proteins from and as genomic RNA. Thus the mutation rate of this enzyme can contribute to HIV mutation rates. The precise mutation rate of human RNA polymerase II has not been established but it is thought that it is probably similar to the mutation rate of the prokaryotic version of the enzyme. This has been calculated as approximately one error every 10^4-10^5 nucleotides synthesised (Blank et al., 1986; Libby and Gallant, 1991). If human RNA polymerase II does have a mutation rate of this order it may play a role in mutation rates of HIV as significant as viral RT.

1.9.1.3 Recombination

Every retroviral virion contains two homologous copies of the RNA genome.

This allows for high rates of recombination; around 2% per kilobase per replication (Hu and Temin, 1990). Recombination is thought to occur mainly during minus-strand DNA synthesis (Zhang et al., 2000). In this model, when the RT encounters breaks in the RNA while synthesising the first strand (minus-strand) of DNA, it can switch to use the other RNA molecule as template. Recombination has been directly observed in a rhesus monkey infected with two strains of SIV each containing distinct mutations which significantly lowered viral replication (Wooley et al., 1997). Within two weeks recombinant virus lacking both mutations predominated. Indirect evidence for recombination also exists in the form of mosaic genomes. A number of CRFs have caused major epidemics (see section 1.3.2). These presumably arose when one cell was infected with two different subtypes of virus. Virus released from this cell could contain a genome from each subtype which could recombine upon proviral synthesis. Discordance of phylogenies between *gag* and *env* sequences from brain and lymph of individual persons suggest that recombination occurs frequently even between anatomically distinct sites (Morris et al., 1999).

1.9.2 Host factors

1.9.2.1 Immune Response

The most important immune factor driving viral evolution seems to be the emergence of CD8+ cytotoxic T lymphocytes (CTL). Unfortunately, within 22-30 days of the emergence of a CTL response, CTL escape mutants can appear and when they do, they rapidly become the dominant variants (Borrow et al., 1997; Price et al., 1997). This provides strong evidence that

host CTL responses are an important driver of evolution. It is thought that the ability of CTLs to control viral load and the emergence of escape mutants are important determinants of the length of the chronic stage of HIV infection.

Antibody responses impose a milder selective pressure on viral evolution than CTL responses. Antibody responses to infection can arise between 5 weeks and a year after initial infection (Arendrup et al., 1992; Pilgrim et al., 1997). Escape mutants to neutralising antibody do appear. In fact, antibody is commonly capable of neutralising virus from earlier time points but not the current viral strains (reviewed in Da Silva 2003). This indicates that there is some advantage to the virus in evolving away from antibody responses. However, the presence of antibody escape mutants in long term non-progressors suggests that their role in disease progression is minimal (Bradney et al., 1999).

1.9.2.2 HLA type

Another factor influencing viral evolution is the human leukocyte antigen (HLA) genotype of the infected person. There are two types of HLA. HLA class I presents intra-cellular antigen to CD8 cells thus generating a CTL response. HLA class II present endocytosed extra-cellular antigen to CD4 cells and thus play a role in cytokine production and T call help. Each class of HLA has three loci each of which can be either homo- or hetero-zygous. Studies investigating specific HLA allelic associations with disease progression have been difficult due to the highly polymorphic nature of the HLA loci. However, some convincing data regarding such associations are emerging. Unsurprisingly, most associations between disease progression

and HLA allele have involved HLA class I as this is involved with generation of CTL responses.

Perhaps the most striking finding in studies of HLA and AIDS is that the degree of homozygosity at class I loci is significantly associated with more rapid disease progression (Carrington et al., 1999; Tang et al., 1999). It is thought that this is because higher diversity of HLA molecules provides a bigger challenge to HIV's attempts to "out-evolve" the immune system. Another factor may be that selective pressure of prevalent HLA alleles has driven HIV variants in the population at large to be resistant to presentation by these (Goulder et al., 1997a; McMichael and Klenerman, 2002). Thus people with a second different allele may be better able to combat the virus.

In addition to this heterozygote advantage, a number of specific HLA alleles with significant associations with disease progression have been identified. Two class I alleles found to be protective against disease progression are HLA-B*57 (Migueles et al., 2000; Klein et al., 1998) and HLA-B*27 (Goulder et al., 1997b). CTL responses to peptides bound to these molecules are strongly protective and emergence of escape mutations is restricted. This is probably because mutations necessary to escape presentation by these HLA alleles negatively affect viral fitness. Conversely the presence of HLA-B*35 is strongly associated with disease progression (Gao et al., 2001). This is partly because this allele is a poor generator of CTL responses but there appears to be a further, as yet uncharacterised, negative effect of this allele (Carrington and O'Brien, 2003).

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1.9.2.3 Anti-retrovirals

Anti-retroviral treatment (although not strictly a host factor) can also exert a strong selection pressure on viral evolution as demonstrated by the rapid emergence of escape mutants following the advent of monotherapy (figure 1.10). The emergence of drug resistant mutants is discussed in more detail in chapter 4.

1.9.3 Models of evolution

For the past fifty years or so there has been much controversy surrounding the predominant mechanism for evolutionary change in general (reviewed in Page and Holmes, 1998). The argument has centred on the relative roles of chance and natural selection on fixing mutations within populations. Two opposing models have been proposed; the neutralist and the selectionist models. Both models are in agreement that the majority of mutations to occur at the molecular level will be deleterious to the organism and will therefore be lost.

The neutralist model states that most other mutations will be neutral (Kimura, 1968; Jukes and Cantor, 1969). The majority of these will also be lost but a small number of these will become fixed. The mutations which become fixed will be a random selection of all possible neutral mutations. This model does accept that some advantageous mutations will occur but these will be so infrequent as to be irrelevant to the overall pattern of evolution. Thus over time the genetic code will change randomly- so called "genetic drift". Selective pressure is a factor in this model but only as a negative pressure to eliminate deleterious mutations.

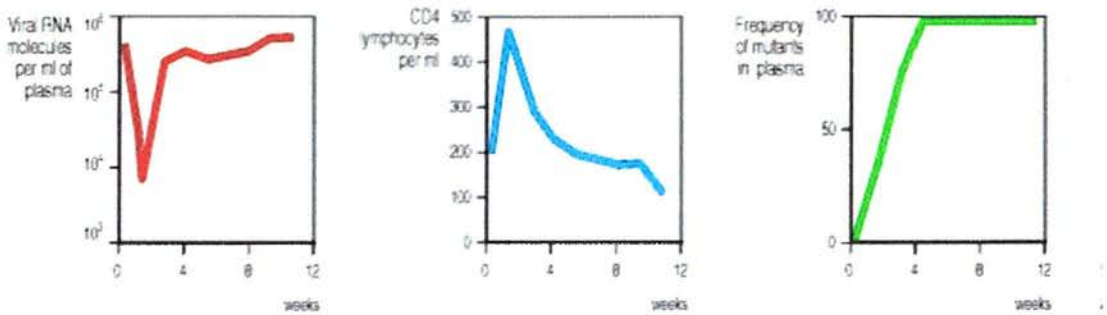


Figure 1.10: Emergence of drug resistance during mono-therapy with a protease inhibitor. Within days of beginning treatment there is a dramatic fall in viral RNA (first graph) and increase in CD4 T cell numbers (second graph). However drug resistant mutants arise quickly leading to a rebound in viral numbers and decline in CD4 T cell numbers. By 4 weeks resistant mutants are the dominant viral variant (third graph) (Wei et al., 1995).

The selectionist model was proposed by some as the obvious extension of Darwinism and gives more weight to positive selection as a factor in evolution. While selectionists also believe that most mutations will be deleterious, they believe that most other mutations will confer at least a slight advantage to the organism. In this model, the mutations which become fixed are those which most improve the fitness of the organism.

Which model best explains evolution in a given circumstance, is determined by a number of factors most important of which seem to be presence of selective pressure and effective population size. If there is substantial selective pressure acting on a gene it may exhibit deterministic evolution characteristic of the selectionist model. This is the case for HIV which is constantly facing changing host pressures. Each time the selection pressure alters, a new opportunity arises for mutations to improve upon viral fitness. When there is a large effective population, survival is much more likely to be determined by fitness whereas in small effective populations random events may be more important in controlling viral evolution. Conflicting reports exist about the effective population size in a HIV infected individual. On the one hand there are a large number of productively infected cells, estimated to be between 10^7 and 10^8 (Haase et al., 1996), and an even larger number of virions generated per day, thought to be at least 10^{10} (Perelson et al., 1996; Rouzine and Coffin, 1999a). However, some studies have suggested that the population of infected cells which produce virus which goes on to infect other cells, is much lower than the total number of infected cells (Leigh-Brown, 1997). This could either be due to frequent bottleneck effects or because

virus produced from most infected cells is actually non-infectious. It has been suggested that the effective population size and therefore relative importance of neutralist or selectionist behaviour, may vary throughout HIV infection depending on external factors (Rouzine and Coffin, 1999b). For example, the genetic bottleneck seen with the advent of anti-retroviral treatment is suggestive of a selectionist model whereas neutralist behaviour may be relatively more important during steady state chronic infection.

1.10 AIDS Dementia Complex

In the absence of effective treatment, there are a variety of neurologic manifestations of HIV infection. A number of brain disorders are caused by opportunistic infections during AIDS such as cryptococcal meningitis, toxoplasma encephalitis, cytomegalovirus encephalitis, neurosyphilis and primary CNS lymphoma. In addition to these, HIV itself can cause neurological dysfunction. The mildest form of HIV-associated CNS disease is termed minor cognitive motor disorder. A more severe disorder, known as AIDS dementia complex (ADC) is associated with significant encephalopathy. The prevalence of this disorder in the absence of effective treatment is controversial; estimates of incidence in persons with AIDS range from 5% to 60% (reviewed in Clifford, 2002; Tardieu and Boutet, 2002). One reason for these disparate results is that it can be difficult to rule out opportunistic infections as the etiological agent for observed clinical deficits. A post-mortem study found that HIV encephalopathy was apparent in 25% of brains from persons with AIDS (Masliah et al., 2000).

1.10.1 Clinical presentation

The clinical presentation of ADC has led to it being classed as a sub cortical dementia. This distinguishes it from so called cortical dementias, such as Alzheimer's disease in which the initial observations include deficits of higher functions including reasoning and memory. In addition to ADC, other diseases including Parkinson's disease- associated dementia and Huntington's chorea are classed as sub cortical dementias. In these illnesses the initial pathological damage tends to be in regions lying below the cerebral cortex although more generalised cerebral pathology may occur with time. Characteristic symptoms include deficits in attention, motivation and emotionality (reviewed in www.memorylossonline.com).

The main features of ADC are sub acute cognitive decline and slowing of motor function (reviewed in Clifford, 2002). Many people with ADC are aware of their own clinical decline. They often report a reduction in concentration span which leads to an impaired ability to perform tasks, read or enjoy hobbies. Loss of motor function is almost ubiquitous in ADC. In fact this symptom alone has been used as a reliable indication of the severity of disease. Motor loss is typically symmetrical and mainly involves the arms and legs. This can result in a reduction of functionality and independence of the affected individual.

1.10.2 Pathological features

The defining features of HIV encephalitis (HIVE), the pathological disorder underlying ADC, are giant cells and or detection of HIV p24 positive microglia/macrophages/giant cells in brain tissue (Budka et al., 1991).

Histological findings may also include astrocytosis, myelin pallor, monocyte infiltration and neuronal loss (reviewed in Bell, 1998; Kaul et al., 2001; Rhodes and Ward, 1991). As suggested above, the earliest pathological changes occur in sub cortical regions. Pathological changes are also present in sub cortical white matter where multinucleated giant cells and abnormal staining are observed (Clifford, 2002). Positron emission tomography scans have revealed hypermetabolism in deep grey structures (Rottenberg et al., 1987). This is followed by marked atrophy of the basal ganglia which has been observed by MRI scans (Aylward et al., 1993).

In later stage ADC, cortical pathology is also apparent. There is a noticeable loss of thickness of the cortical mantle. The reason for this loss may vary by brain region. In the hippocampus, atrophy of neurons without cell loss was observed (Sa et al., 2000). However, quantitative neuronal loss is apparent in the frontal and temporal lobes (Everall et al., 1991). The mechanism for this cell loss is thought to be apoptosis mediated by a number of mechanisms outlined below. In addition, other more subtle pathological changes such as synaptic changes, vacuolisation and β–amyloid accumulation can occur (Giometto et al., 1997; Masliah et al., 1997).

Surprisingly, neither the viral titre in the brain, nor most of the pathological changes outlined above, correlate well with the clinical severity of ADC. In fact the best pathological indicator of degree of impairment is the amount of macrophage activation (Glass et al., 1995; Adle-Biassette et al., 1999).

1.10.3 Pathogenesis

The only brain cells known to be productively infected by HIV are microglia

however the current model of neuropathogenesis involves interactions between HIV, microglia, astrocytes and neurons. This model is summarised in figure 1.11 and the role of the different cell types is outlined below.

Endothelial Cells

In order to enter the brain HIV must get past the endothelial cells which line the blood brain barrier (BBB). Some studies have suggested that HIV achieves this by first infecting endothelial cells; however subsequent studies have disputed this (Nottet et al., 1996). An alternative hypothesis is that HIV or HIV proteins are toxic to endothelial cells and thus increase the permeability of the BBB. Toxicity of gp120 and gp160 proteins has been demonstrated for both rat and human brain endothelial cells (Kanmogne et al., 2002). Supporting this theory is the observation of apoptotic endothelial cells in post mortem brain sections from individuals with AIDS (Shi et al., 1996).

Microglia and macrophages

Infiltration of infected macrophages is thought to be the primary route of HIV entry into the brain (reviewed in Gartner, 2000) and macrophages are also the principal site of viral replication within the brain. A number of types of macrophage can exist in the brain and these may vary in their ability to support HIV infection. As mentioned above, blood monocytes can enter the brain and within the brain there exist microglia. In addition, brain macrophages reside in the meninges and choroid plexus. It is thought that each of these cell types must be activated before they are permissive to HIV infection (reviewed in Tardieu and Boutet, 2002). Soluble gp120 alone

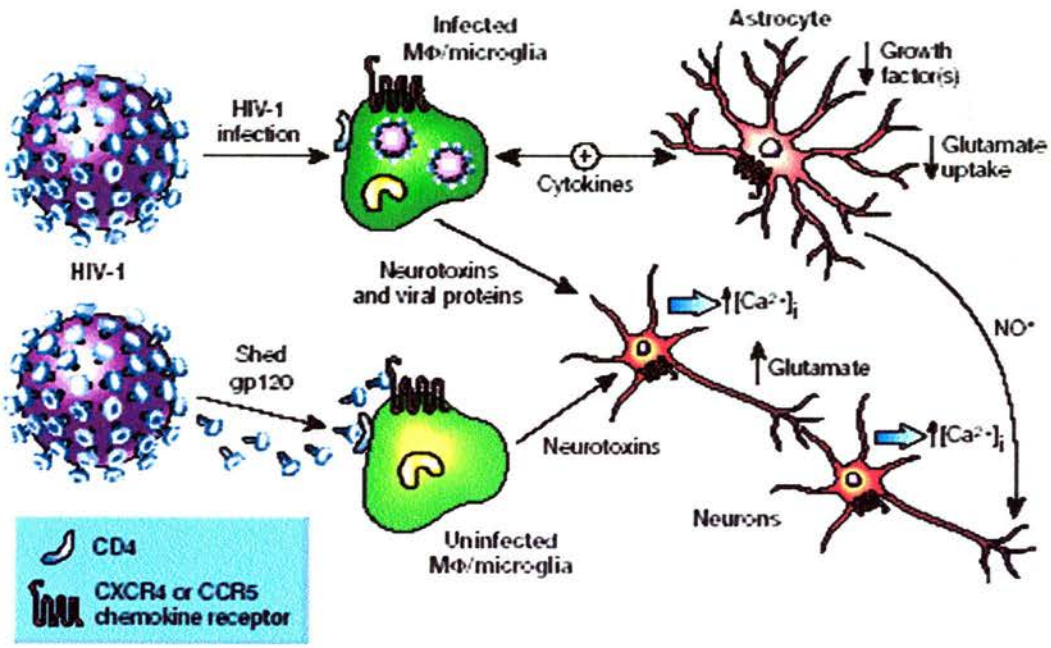


Figure 1.11: Model of pathways leading to neural damage in HAD. A cascade of damaging effects is initiated either by infection of macrophages/ microglia or by the effects of shed HIV proteins. Substances released from macrophages and microglia can have a direct toxic effect on neurons. Additionally, some substances can cause astrocytes to release neurotoxic substances (Kaul et al., 2001).

can also bind and stimulate macrophages or microglia.

Once activated/ infected the cells release various damaging substances which have effects on other cells. In fact infected and/or activated macrophages/microglia are necessary for HIV or gp120 induced neuronal damage (Giulian et al., 1990; Giulian et al., 1993). Neuronal damage can be mediated by the release of excitotoxic substances such as glutamate, inflammatory-cytokines such as IL1-β and TNF-α, cytokine-induced L-cysteine, arachidonic acid, chemokines, free radicals such as superoxide anion and HIV-proteins (Lipton and Gendelman, 1995; Giulian et al, 1990; Pulliam et al., 1991).

Astrocytes

Astrocytes may be non-productively infected with HIV (reviewed in Brack-Werner, 1999; Tornatore et al., 1994; Ranki et al., 1995). As astrocytes play a role in inactivating neurotoxins (Fine et al., 1996), buffering glutamate and maintaining the blood-brain barrier (Brack-Werner, 1999); this could have an influence on neuropathogenesis. One study has reported that a high level of HIV DNA-positive astrocytes is associated with higher levels of astrocyte apoptosis and a more rapid disease progression (Thompson et al., 2001).

Microglial released substances such as TNF-α and arachidonate can act on astrocytes both to induce glutamate release and to inhibit glutamate reuptake (Fine et al, 1996; Genis et al., 1992). In addition cytokines and viral proteins can act on inducible nitric oxide synthase (iNOS) in astrocytes. The resultant nitric oxide is a modest neurotoxin however it can also react with microglial released superoxide anion to produce the potent oxidant peroxynitrite

(Beckman and Koppenol, 1996).

Neurons

While there is no evidence of productive infection of neurones with HIV, there is conflicting data on the frequency and significance in the aetiology of HIV-related brain disease of latent infection in this cell type (Nuovo et al., 1994; Bagasra et al., 1996; An et al., 1999).

Various *in vitro* studies have found evidence for direct neurotoxic effects of HIV-proteins (Meucci et al., 1998; Hesselgesser et al., 1997). However the significance of these findings *in vivo* is unclear. The concentrations of viral proteins used experimentally often far exceed those found in HIV-infected brains and experiments using mixed neural cell cultures have shown that indirect effects of microglial and astrocytic released substances may play a more significant role in neural damage (Lipton and Gendelman, 1995; Kaul and Lipton, 1999).

Excitotoxic substances such as glutamate released from microglia and astrocytes can over-activate glutamate receptors, particularly the N-methyl-D-aspartate (NMDAR) subtype. Severe over-activation can cause the cell to become necrotic while a lower degree of activation can initiate the cellular pathways which result in apoptosis (Bonfoco et al., 1995).

TNF-α and IL-β released from microglia stimulate production of L-cysteine which can also stimulate NMDARs and lead to neuronal apoptosis (Yeh et al., 2000). It is thought that TNF-α may act synergistically with HIV/Tat to induce neuronal death (Shi et al., 1998).

Finally free radicals are thought to play a part in neuronal damage. In particular, peroxynitrite is able to disrupt neurofilament assembly (Beckman and Koppenol, 1996; Coyle and Puttfarcken, 1993).

1.11 Tropism

The cells known to be infected by HIV *in vivo* are T cells, macrophages, microglia and dendritic cells. The infection of a number of other cells such as astrocytes and endothelial cells remains controversial (Brack-Werner, 1999; Bagasra et al, 1996; An et al, 1999). This relatively narrow tropism reflects HIV's requirement for both CD4 and a co-receptor in order to infect cells (Deng et al., 1996; Feng et al., 1996) (section 1.4.1). A number of viral and cellular factors contribute to the differential tropism of various viral strains for different cell types.

1.11.1 Co-receptors

The discovery that HIV uses seven transmembrane chemokine receptors as co-receptors led many to believe that the issue of HIV tropism would be solved. However, while around 15 chemokine receptors have been found to support HIV infection *in vitro*, it appears that only 2 are used significantly *in vivo* (reviewed in Clapham and McKnight, 2001). CCR5 is the main receptor used during transmission and asymptomatic infection (Benkirane et al., 1997; Wilkinson et al., 1998; Scarlatti et al., 1997). With the onset of AIDS, there is a switch to CXCR4 usage in some people- around 50% of those infected with subtype B (Scarlatti et al, 1997). In some study subjects dual tropic (X4R5) viruses are found. CCR5 using (R5) viruses are able to infect memory T cells and macrophages while CXCR4 using (X4) viruses are able to infect both

naïve and memory T cells and some populations of macrophages (reviewed in Clapham and McKnight, 2001). More subtle differences in tropism than merely co-receptor usage exist. For example although macrophage tropism has been used interchangeably with "R5" this may be misleading. In one study both non-macrophage tropic R5 viruses and macrophage tropic X4 viruses were identified (Gorry et al., 2001). Another paper reported that CCR5 deficient macrophages allowed CXCR4 dependent entry by a dual tropic virus but not by an X4 virus (Collman and Yi, 1999). These results suggest that macrophage tropism is not solely determined by co-receptor usage.

1.11.2 Receptor expression levels

In some cases the relative levels of receptor expression may influence tropism. The inability of primary X4 viruses to replicate in T cell lines was overcome by overexpression of CD4 (Platt et al., 2000) while over-expression of CXCR4 on primary macrophages dramatically increased the efficiency of infection by X4 laboratory isolates (Tokunaga et al., 2001). However, receptor expression levels cannot fully explain differences in tropism. Altering the expression levels of either CD4 or CCR5 did not explain the differences in tropism between a macrophage tropic and a non-macrophage tropic R5 isolates (Gorry et al, 2001).

1.11.3 Other receptors

While CD4 and a co-receptor are required for viral fusion, it is possible that other receptors may cause initial cell adhesion and thereby influence tropism.

When HIV buds from a cell it selectively incorporates certain host cell derived

membrane molecules (Tremblay et al., 1998; Henriksson and Bosch, 1998). The nature and quantity of adhesion molecules acquired may influence the cell type adhered to and subsequently infected by the virion. Virus grown in cells overexpressing intercellular adhesion molecule 1 (ICAM-1), had a 10 fold increased infectivity for LFA-1 cells (Fortin et al., 1997).

A number of other cell-surface molecules may contribute to attachment. Heparan sulfate proteoglycan (HSPG) expression can greatly affect the infection of cells by laboratory X4 viruses (Mondor et al., 1998; Roderiquez et al., 1995; Patel et al., 1993) although its significance *in vivo* is unclear. Other molecules which could potentially play a role include mannose receptors and galactosyl ceramide (Ugolini et al., 1999).

1.11.4 Viral Factors

The envelope protein of HIV consists of a three transmembrane gp41 peptides, non-covalently linked to three extra-cellular gp120 peptides. Gp120 consists of 5 conserved and 5 variable regions (figure 1.12). Particularly important to determining tropism is the V3 loop (Kwong et al, 1998). although V1 and V2 also have some influence (Cho et al., 1998). The V3 loop itself has two functional domains: the V3 crown and the V3 loop. Both the crown and loop region appear to play a role in the binding of soluble R5 gp120 to CCR5 however only the crown region is required for CCR5 binding by virion associated gp120 (Cormier and Dragic, 2002). The genetic factors which determine co-receptor usage are variable and strain specific. An association between the presence of basic residues in V3 and T-cell tropism has been noted (Fouchier et al., 1992; Basmaciogullari et al., 2002). Substitutions at a

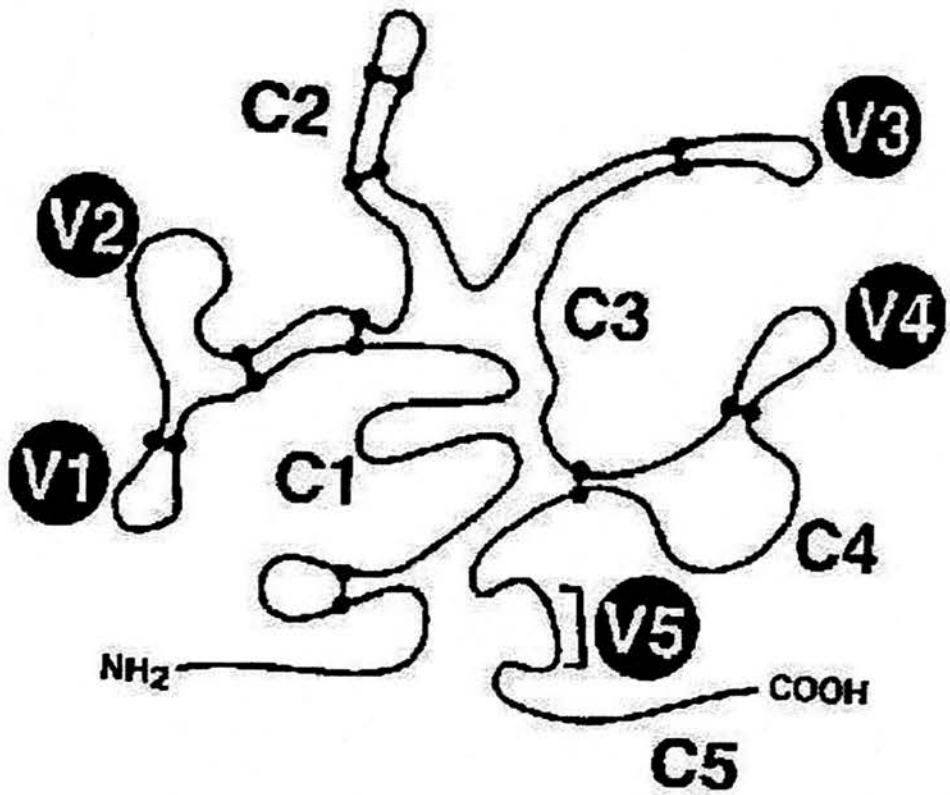


Figure 1.12: Schematic representation of the variable (V1-V5) and conserved (C1-C5) regions of gp120 (Hoffman and Doms, 1999).

number of amino acid sites in gp120 are correlated with a switch from CXCR4 to CCR5 usage *in vivo* (Rizzuto et al., 1998).

1.12 Neurotropism

1.12.1 Compartmentalisation

Within an infected individual, the *env* gene from brain or lymphoid tissue derived virus can differ significantly. This mirrors similar differences observed between lymph derived virus and that found in semen, faeces or lungs. One study found that virus from the colons and brains of patients grouped together phylogenetically and were distinct from blood-derived virus (Wang et al., 2001). This suggests that rather than there being many separate tissue compartments, a population of virus arises which is able to infect tissue macrophages in many different organs.

There are two possible explanations for the differences observed in viral sequences derived from the blood/lymphoid tissue and those from the brain. It could be that these populations are physically separated from each other and thus viral evolution occurs independently in each. Alternatively, the differences could reflect an adaptive process whereby the virus evolves to be better able to infect the target cells in the tissue in which it is located. Accumulating evidence suggests that there is no physical barrier between the blood and the brain viral compartments. One study showed that the segregation of viral populations between the intestinal and blood compartments is not complete (Vanderhoek et al., 1998). Rather, while the proportion of two variants differed in the two compartments, both variants could be found to some degree in both compartments. This shows that there

is no physical impediment to variants mixing between these two compartments. Further evidence that differences in viral populations between blood and brain are due to adaptive processes was provided by a study which examined recombination between variants in these two locations (Morris et al, 1999). The presence of discordant phylogenies for different HIV genes when sequences for the two populations were compared led to the conclusion that the viral subspecies infecting brain and lymphoid cells recombine frequently. This suggests that there is no barrier to recombination between compartments allowing some genes (especially *env*) to evolve to adapt to the new environment, while others can retain similarity to those found elsewhere in the body via recombination.

1.12.2 Neurotropic versus macrophage tropic

Specifically neurovirulent SIV_{MAC} strains have been isolated (Anderson et al., 1993) but whether such variants exist for HIV is controversial. One study found that brain-derived virus showed a preferential tropism for microglia compared to blood monocytes-derived macrophages (Strizki et al., 1996). However others have found that microglia and macrophage tropism of brain-derived virus is similar (Ghorpade et al., 1998; Hibbitts et al., 1999).

1.12.3 Co-receptors in the brain

When considering infection of cells in the brain it is important to determine the cellular distribution of receptors able to mediate HIV infection (reviewed in Martin-Garcia et al., 2002). The principle resident brain cells infected are microglia. These express CCR5 as well as low levels of both CD4 and CXCR4 (Dick et al., 1997; He et al., 1997; Albright et al., 1999). In addition a

number of other potential coreceptors such as CCR3 are found on this cell type. CCR5 seems to be the main coreceptor used for infection of these cells as anti-CCR5 antibodies are able to inhibit infection *in vitro* (Albright et al, 1999).

The other brain cell type for which convincing evidence of infection exists are astrocytes. A number of studies have found evidence of HIV infection of astrocytes *in vivo* (reviewed in Brack-Werner, 1999) although there have been questions about the validity of some techniques used. Certainly primary human foetal astrocytes can be non-productively infected *in vitro* (Sabri et al., 1999). The cellular receptors which mediate infection of this cell type remain something of a mystery. These cells do not appear to express CD4 at all and express CCR5 and CXCR4 only at very low levels. Moreover, neither antibodies to the CD4 binding site of HIV or to chemokine receptors, nor a variety of chemokines, inhibited infection of primary human foetal astrocytes (Sabri et al, 1999). If these results reflect the *in vivo* situation, it would mean that astrocytes are the first identified CD4 negative cell type to be infected *in vivo*. This surprising possibility is probably the reason that the scientific community has been slow to accept that astrocytes are infected at all. The receptor (s) which does mediate astrocyte infection *in vitro* or *in vivo* remains uncharacterised. There has been some evidence that the glycosphingolipid galactosylceramide (GalCer) could mediate infection of this cell type (Harouse et al., 1991; Bhat et al., 1991) however this is still controversial.

Aims

The aim of this project was to investigate the distribution of HIV within its host.

Each results chapter focuses on a different aspect of this topic. The first chapter aims to produce a system which would allow the investigation of the determinants of cellular tropism. The second chapter seeks to characterise the distribution of drug-resistance mutations in different anatomical locations. The final chapter attempts to identify the cellular location of HIV within the central nervous system. Thus the project attempts to further the understanding of where HIV is found, determinants of cellular tropism and how the virus differs phenotypically in different locations.

Chapter 2: Materials and methods

2.1 Analysis and manipulation of DNA

2.1.1 Minimising contamination in PCR

Nested polymerase chain reaction (PCR) is a highly sensitive technique which can be used to amplify DNA sequences from only one template molecule. This sensitivity makes nested PCR an extremely valuable tool in many aspects of molecular biology research however is also means that there is a high risk of contamination which could lead to false positive results.

A number of steps were taken to reduce the risk of DNA contamination. All PCR buffers were mixed in a dedicated room which was never used for nucleic acid manipulations. Primary and secondary PCRs were set up in separate rooms with dedicated equipment (e.g. pipettes) and lab coats. Equipment was never transferred from the secondary lab to the primary lab. Likewise, once a primary PCR had been carried out, the PCR tubes were never opened in the primary lab. Throughout procedures latex gloves were used. These were always removed before leaving a lab. DNA contamination was minimised within all labs by regularly cleaning lab benches and pipettes with a solution of 1% (v/v) bleach.

Known negative control DNA samples were included along with every PCR run to check for any DNA contamination.

2.1.2 Primer sequences

A number of considerations were taken into account when designing PCR primers. The portion of DNA sequence to be used as a template for primer design was carefully chosen to yield optimal primers. A region was chosen which had a G + C content of 40-60%, did not contain runs of any one base

and did not contain "pallendromic" sequences which could lead to secondary structure formation of the primers. Primers were generally designed to be between 20 and 30 bases long and had a high degree of sequence similarity with the template DNA of interest. In particular, mismatched bases were avoided at the 3' end of primers as this can substantially reduce primer binding. Complementary sequences between primers were avoided in order to reduce primer dimmer formation. Oligonucleotide primers were synthesised by VH Bio or Oswell. Primer sequences used are outlined below in table 2.1.

Name	Primer sequence (5' to 3')	Function
EGFPS	GTACCCCATGGTCTCGAGTGAGCAAGGGCGAG GAG	EGFP S
EGFPAS	TTACTTGTACAGCTCGTCCAT	EGFP AS
EGFPSKpn	CCGGGTACCCCATGGTCTCGAGTGAGCAAGGG CGAGGAG	EGFP S (incorporates Acc 65I site)
EGFPASBsi	CGAACGTACGTTACTTGTACAGCTCGTCCAT	EGFP AS (incorporates Bsi WI site)
M13S	TTGTAAAACGACGGCCAGTG	Fragment for OE
M13AS	GGAAACAGCTATGACCATGA	Fragment for OE
332	TACAATGTACACATGGAATT	V3 OS
306	TGGCAGTCTAGCAGAAGAAG	V3 IS
307	CTGGGTCCCCTCCTGAGG	V3 IAS
308	ATTACAGTAGAAAAATTCCCC	V3 OAS
531	GCGAGAGCGTCAGTATTAAGCGG	p17 OS
532	GGGAAAAAATTCGGTTAAGGCC	p17 IS
533	CTTCTACTACTTTTACCCATGC	p17 IAS
534	TCTGATAATGCTGAAAACATGGG	p17 OAS

291	N/K	β-actin OS
293	N/K	β-actin IS
294	N/K	β-actin IAS
292	N/K	β-actin OAS
Env A	GGCTTAGGCATCTCCTATGGCAGGAAGAA	Env OS
Env B	AGAAAGAGCAGAAGACAGTGGCAATGA	Env IS
Env M	TAGCCCTTCCAGTCCCCCCTTTTCTTTTA	Env IAS
Env N	CTGCCAATCAGGGAAGTAGCCTTGTGT	Env OAS
P51OS	TTAGTAGGACCTACACCTGTC	RT OS
P51 OAS	CCCCACTAACTTCTGTATGTC	RT OAS
PA IS	GGAATGGATGGCCCAAAAGTT	RT (A) IS
PA IAS	CTGATATCTAATCCCTGGTGTCTC	RT (A) IAS
PB IS	CTGGATGTGGGTGATGCATAT	RT (B) IS
PB IAS	CACTATAGGCTGTACTGTCCA	RT (B) IAS
PROF	ACCAGAAGAGAGCTTCAGG	PR OS
PRIF	AACTCCCYCTCAGAAGCAGG	PR IS
PRIR	TGGTACAGTTTCAATAGGAC	PR IAS
PROR	TTGGGCCATCCATTCCTGGC	PR OAS

Table 2.1: Oligonucleotide primer sequences and function. OS: Outer sense; IS: Inner sense; IAS: Inner anti-sense OAS: Outer anti-sense; N/K: Primer sequence not known as primers obtained from colleague with no record of sequence.

2.1.3 Basic PCR reactions

PCRs were carried out using a Genius PCR machine (Techne). PCRs were carried out in reaction volumes of 20 µl unless specified. Reaction mix consisted of 2 µl template DNA (0.5 µl if template was cloned plasmid DNA), 10% (v/v) 10 x Promega PCR reaction buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 15 mM MgCl2 and 1% (v/v) Triton X-100), 30 µM of each dNTP (dGTP, dATP, dTTP and dCTP) (Promega), 2 units of *Taq* polymerase (Promega), 0.25 µM of each primer and nuclease free water (Promega) to

the final reaction volume.

The thermocycling conditions used were as follows:

For V3, PR, p17 and β-actin (both primary and secondary):

[94°C for 18 seconds, 55°C for 21 seconds and 72°C for 1.5 minutes] for 30 cycles followed by 72°C for 10 minutes

For RT primary:

[94°C for 18 seconds, 60°C for 21 seconds and 72°C for 1.5 minutes] for 40 cycles followed by 72°C for 10 minutes

For RT secondary:

[94°C for 18 seconds, 60°C for 21 seconds and 72°C for 1.5 minutes] for 25 cycles followed by 72°C for 10 minutes

When nested PCR was required, 2 µl of the primary PCR reaction mix was transferred to a clean tube and acted as template for the secondary reaction. The basic recipe used for the reaction mix was the same for both primary and secondary reactions.

2.1.4 Long range PCR

PCRs were carried out using a Genius PCR machine (Techne). Long range PCRs were carried out in reaction volumes of 50 µl. Reaction mix consisted of 2 µl template DNA (0.5 µl if template was cloned plasmid DNA), 10% (v/v) 10 x Roche Expand High Fidelity PCR reaction buffer with $MgCl_2$, 60 µM of each dNTP (dGTP, dATP, dTTP and dCTP) (Promega), 2 units of Expand

High Fidelity polymerase (Roche), 0.5 µM of each primer and nuclease free water (Promega) to the final reaction volume. The thermocycling conditions used were:

[94°C for 45 seconds, 50°C for 45 seconds and 72°C for 4 minute] for 20 cycles followed by [94°C for 45 seconds, 50°C for 45 seconds and 72°C for 4 minutes 15 seconds] for 15 cycles followed by 72°C for 10 minutes.

When nested PCR was required, 2 µl of the primary PCR reaction mix was transferred to a clean tube and acted as template for the secondary reaction. The basic recipe used for the reaction mix and the thermocycling conditions were the same for both primary and secondary reactions.

2.1.5 Limiting dilution PCR

Serial ten fold dilutions of the extracted DNA ranging from 10^{-1} to 10^{-5}, were prepared using nuclease free water (Promega). Nested PCRs were carried out using 2µl of each dilution as template. 20 replicate nested PCRs were carried out using DNA at a dilution of 1/3 of the last positive dilution. If eight or fewer of these PCRs were positive then the products were assumed to be derived from a single template molecule. If more than eight reactions were positive then a further 20 reactions were carried out at a new dilution which would be expected to yield eight or fewer positives out of 20.

The average number of template molecules (T) present per tube was calculated from the proportion of positive reactions (p) using the formula below:

$$T = -\ln(1-p)$$

The total DNA concentration of samples was also assessed using a spectrophotometer and thus the number of proviral copies per million cells was calculated on the basis that a human diploid cell contains 6.6 pg of DNA.

2.1.6 Generation of fragments by overlap extension

Amplification of fragments was carried out in a 50 µl reaction volume using either 5 µl of ligation mix or 1 µl of NL4.3envEGFP plasmid. The primers used for amplification were M13S and 307 for one fragment and 306 and M13AS for the second fragment. Fragments were generated by thermocycling using the following conditions:

[94°C for 45 seconds, 50°C for 45 seconds and 72°C for 6 minutes] for 35 cycles followed by 72°C for 10 minutes

Products were visualised on an ethidium bromide stained agarose gel to ensure fragments of the predicted size were present. 10 µl of each fragment were mixed. Primers were removed from this mix either by spin purifying or by adding 4 µl exonuclease I (Amersham) and incubating at 37°C for 20 minutes. If the sample was treated with exonuclease heat inactivation of the enzyme at 80°C for 15 minutes was carried out. To the mixture was added the follow:

10% (v/v) of total final volume 10 x Taq reaction buffer (n.b. exonucleased samples will contain buffer from PCR step and only the excess amount required was added), 1 µl Taq, 1 µl dNTPs (Promega), 0.5 µl primer M13S and 0.5 µl primer M13AS

Thermocycling was carried out as follows:

[94°C for 45 seconds, 72°C for 7 minutes] for the number of cycles indicated

2.1.7 Automated sequencing

Automated sequencing reactions were carried out using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed using an ABI Prism 3100 genetic analyser (Perkin Elmer ABI biosystems). This system uses the chain termination method (Sanger et al., 1977) of cycle sequencing (Murray, 1989). The reaction mix includes the thermostable polymerase AmpliTaq DNA polymerase, reaction buffer, dNTPs and terminating nucleotide analogues. These nucleotide analogues are labelled with fluorescent dyes. Each of the four types of nucleotide analogue is labelled with a unique colour of fluorescent dye. Thermocycling the reaction mix with a DNA fragment results in the production of single strands of DNA of various lengths labelled with the colour of the terminating nucleotide. These can be analysed using the genetic analyser to obtain the sequence of the fragment.

For each fragment to be analysed two sequencing reactions were carried out: one using a sense primer located at the 5' end of the fragment and one using an anti-sense primer located at the 3' end of the fragment. To sequence the RT region two overlapping fragments were obtained and sequenced for each product. The primers used for sequencing different fragments are shown in table 2.2.

DNA fragment	Sense primer	Anti-sense primer
V3	306	307
RT (frag A)	PA IS	PA IAS
RT (frag B)	PB IS	PB IAS
PR	PRIF	PRIR

Table 2.2: Primers used for sequencing reactions

3 μl of BigDye reaction mix was added to approximately 50 ng of DNA and the mix made up to 10 μl with nuclease free water (Promega). The mixture was overlaid with a drop of mineral oil. Thermocycling conditions were as follows:

[96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes] for 25 cycles

After thermocycling, reaction was held at 4°C until it was transferred to a 1.5 ml eppendorf tube and sent for automated sequencing.

2.1.8 Sequence analysis

All manipulations and alignment of sequences was carried out using the Bioedit sequence alignment editor computer package (Hall, 1999). Sequences were converted from ABI to FASTA format for analysis. Sequences obtained using reverse primers were converted to their reverse complement form. The "forward" and "reverse" sequences obtained for a given region were compared. Ambiguous loci or loci at which discordant bases appeared in the two sequences were identified. The fluorescence trace file was examined and the base which appeared most probable chosen.

In this way, a single sequence was obtained for each PCR product. Sequences were aligned manually. Attempts were made to minimise the number of insertion/deletions required to fully align sequences. Where an insertion or deletion was necessary, it was located in such a way as to minimise disruption to the amino acid sequence. Where significant differences from the rest of the alignment were noted, the trace file was re-examined to ensure that the sequence was not due to an artefact.

Phylogenetic trees were created from the aligned sequences using the Molecular Evolutionary Genetic Analysis (MEGA) software package (version 2.1). The programme was used to generate a distance matrix for the sequences and then to convert them into a neighbour-joining tree. The observed number of nucleotide differences between two sequences under-represents the "true" genetic divergence as it does not take into account multiple substitutions at the same site; this bias becomes more pronounced as sequences diverge further. There are a number of different distance correction methods which estimate "true" genetic divergence from observed genetic difference. Multiple HIV sequences obtained from a single individual will differ at relatively few bases and thus the simple one parameter Jukes-Cantor distance correction method was used to generate a distance matrix. In this model, the likelihood of any nucleotide being substituted by any other nucleotide is assumed to be equal. The estimated number of nucleotide substitutions (K) is calculated from the observed number of nucleotide differences (λ) using the equation below:

$$K = -3/4\log(1- 4/3\ \lambda)$$

The distance matrix was used to produce a tree using the neighbour-joining method. This method uses a complex algorithm to produce an estimate of the minimum evolution tree (i.e. the tree which fits the data with the shortest total branch length). An outgroup of epidemiologically unlinked sequences was included to allow the tree to be rooted.

To test the robustness of the phylogenetic relationships revealed by the tree, bootstrap re-sampling was employed. In this method, "pseudoreplicate" sequences are produced by sampling (with replacement) basepairs from the sequence data used for construction of the tree. The alignment of pseudoreplicate sequences is used to create a new tree and any splits which are common to this and the original tree noted. This process is repeated many hundreds of times. The bootstrap value of a given split in the original tree is the percentage of the generated trees which contained the same split. Bootstrap values of greater than 70% were displayed on the trees.

2.1.9 DNA quantification using spectrophotometer

The concentration and approximate purity of DNA samples were assessed by taking spectrophotometric absorbance readings at wavelengths of 260 nm and 280 nm. The spectrophotometer used was a GeneQuant II (Pharmacia Biotech). The concentration of DNA in μg/μl was calculated by multiplying the optical density at 260 nm by 0.05. The ratio of optical density at 260 nm to optical density at 280 nm was used as an indication of purity. Pure DNA has a 260:280 ratio of approximately 1.8.

2.1.10 Visualisation of DNA on agarose gel

10 x TAE buffer was prepared by dissolving 242 g Tris base (AnalaR), 57.1

ml Glacial Acetic acid (Molecular Biology certified; Kodak) and 100 ml 0.5 M EDTA (Molecular Biology certified; Kodak) in distilled water to a final volume of 1 litre. pH was adjusted with dilute HCl to 8.5. 1% (w/v) agarose gels were made by adding 3 g of agarose (Sigma) to 300 ml of 1 x TAE buffer and dissolving by heating to boiling point in a microwave. Mixture was allowed to cool to approximately 50°C before the addition of 20 µl of 10 mg/ml ethidium bromide (Sigma). Gel was poured into flat bed mould into which combs had been inserted. Gel was allowed to set for at least 1 hour before use.

DNA samples were mixed in a ratio of 5:1 with 6 x loading buffer (50% (v/v) glycerol, 7.5% (w/v) Ficoll 400, 0.015% (v/v) bromophenol blue, 0.015% (v/v) xylene cyanol, 0.2% (v/v) orange G, 5 mM Tris-HCl (pH 7.5) and 50 mM EDTA. Gells were submerged in 1 x TAE and DNA/loading buffer mix pipetted into the wells. A voltage of 150 V was applied across the gel for between 30 minutes and 2 hours depending on the expected size of the DNA fragments. Bands of DNA were visualised by the fluorescence of bound ethidium bromide under a shortwave (254 nm) ultra violet light. 100 bp or 1 kb DNA step ladder (Promega) were run along side samples to allow assessment of DNA fragment size.

2.1.11DNA purification using QIAquick PCR purification kit (Qiagen)

DNA cleanup was routinely carried out using QIAquick PCR purification kit. This kit makes use of the fact that DNA will bind to a silica membrane in the presence of a high salt concentration but will be eluted in low salt conditions. All centrifugation steps were carried out at 12000 x g for 1 minute at room

temperature. 5 volumes of buffer PB were mixed with 1 volume of DNA sample and the mixture added to a QIAquick spin column. The column was centrifuged. The high salt concentration of the buffer allows the DNA to bind to the silica membrane while impurities and liquid pass through. 0.75 ml of buffer PE, a high salt concentration wash buffer containing ethanol was added to the column and the tube centrifuged. The flow through was discarded and the tube re-centrifuged to remove residual buffer. The column was placed in a clean tube and 30 µl nuclease free water (Promega) added to the membrane. The column was incubated at room temperature for 1 minute before centrifuging it to collect eluted DNA.

2.1.12 DNA extraction from agarose gel using QIAquick Gel Extraction Kit (Qiagen)

DNA was extracted form agarose gels using the QIAquick Gel extraction kit. The columns used in this kit are identical to those used for DNA cleanup. The band of DNA of interest was excised from the gel and placed in a clean eppendorf. The fragment of gel was dissolved at 50°C for 10 minutes in a volume in mls three times the weight in grams of the gel fragment of buffer QE. This mixture was added to the column and the column centrifuged. From this point, the DNA was washed and eluted as described above for DNA cleanup.

2.1.13 Enzymatic manipulations of DNA

Restriction digests

Restriction digests were carried out in total volumes between 20 and 100 µl. The reaction mix consisted of DNA to be digested, 10% (v/v) of the appropriate New England Biolabs or Promega 10 x buffer, 0.1 µl/ml bovine

serum albumin (BSA) (Promega), approximately 1 unit of restriction enzyme per µg DNA and nuclease free water (Promega) to the final volume. Where double digests were performed, a buffer which allowed both enzymes to digest adequately was selected. Digests were allowed to proceed for at least 1 hour at 37°C.

Shrimp alkaline phosphatase

Shrimp alkaline phosphatase (SAP) (Amersham) was used to remove phosphate groups from the 5' strand of digested plasmid. This prevents the plasmid from self-ligating. Following digestion, restriction enzymes were heat inactivated as specified for individual enzymes by manufacturer (usually 80°C for 15 minutes). Reaction was cooled to room temperature before addition of 1 µl of SAP per 50 µl of reaction mix. Reaction was incubated at 37°C for 15 minutes and then heat inactivated at 80°C for 15 minutes.

Ligations

Relative concentrations of vector and insert were estimated by visualisation of DNA on an agarose gel. Vector was mixed with insert at a molar ratio of approximately 1:2 to a total volume of 8 µl. 1µl of 10 x ligation buffer and 1 µl of T4 DNA ligase (NEB) were added and the liquid mixed by pipetting. Reaction was allowed to proceed at room temperature overnight.

2.2 Recombinant DNA technology

2.2.1 Reagents

SOC media: 2% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Gibco), 0.005% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM glucose in distilled water

Luria-Bertani (LB) Broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl in distilled water

LB agar plates: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% NaCl (w/v) and 1% (w/v) agar in distilled water

Competent cells: For all transformations Select 96™ Competent Cells (Promega) were used.

2.2.2 Transformation

Competent cells were stored at -70°C. A vial containing 50 µl competent cells was thawed on ice (approximately 5 minutes). 5 µl of ligation reaction was added and the cells mixed gently by flicking the tube. Cells were incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds and then placed back on ice for 2 minutes. 250 µl SOC media was added and the vial was transferred to a 37°C incubator with orbital shaking (225 rpm) for 1 hour. LB agar plates containing 50 µl/ml ampicillin were pre-warmed in a 37°C incubator. Aliquots of 50 µl and 200 µl of cells were spread evenly over separate plates using a sterile glass spreader. Plates were left at room temperature for 30 minutes before being inverted and placed in a 37°C incubated overnight.

2.2.3 Minipreps of plasmid DNA

3 ml of LB broth containing 1 μg/μl ampicillin (Sigma) was poured into a sterile 15 ml tube and inoculated with a single colony of bacteria. The culture was incubated at 37°C or 30°C as indicated for 16 hours with orbital shaking (225 rpm). Bacterial cells were pelleted by centrifugation at 6000 x g for 3 minutes. Plasmid DNA was extracted using the QIAprep Miniprep kit (Qiagen). The procedure involves alkaline lysis of the bacterial cells followed by adsorption of plasmid DNA to a silica membrane in the presence of a high salt concentration. The DNA can be eluted from the membrane by a low salt buffer.

Bacterial cell pellet was resuspended in 250 μl of Buffer P1 and transferred to a 1.5 ml eppendorf tube. 250 μl of buffer P2 was added and the tube gently inverted 6 times. 350 μl of buffer N3 was added and the tube mixed by inverting gently 6 times. The tube was centrifuged for 10 minutes at 12000 x g to pellet the white precipitate (containing cellular matter and genomic DNA). The supernatant was transferred to a QIAprep column placed in a 2 ml collection tube. The tube was centrifuged at 6000 x g for 1 minute and the flowthrough discarded. 500 ml of buffer PB were added. The tube was centrifuged at 6000 x g for 1 minute and the flowthrough discarded. This step efficiently removes endonucleases. 750 μl of buffer PE were added. The tube was centrifuged at 6000 x g for 1 minute and the flowthrough discarded. This step removes salts. The tube was centrifuged at 6000 x g for an additional 1 minute to thoroughly dry the membrane.

The column was transferred to a clean 1.5 ml eppendorf tube. 50 μl of

nuclease free water (Promega) was added to the centre of the membrane. The tube was allowed to sit at room temperature for 1 minute. The tube was then centrifuged at 6000 x g for 1 minute to elute the DNA in the water.

2.2.4 Midipreps of plasmid DNA

3 ml of LB broth containing 1 μg/μl of ampicillin was poured into a sterile 15 ml tube. The broth was inoculated with a single bacterial colony and incubated at 30°C or 37°C, as indicated in text, for approximately 8 hours. 200 μl of this started culture was added to a sterile flask containing 100 ml LB broth containing 1 μg/μl ampicillin. This culture was incubated at 30°C or 37°C, as indicated in text, for approximately 16 hours with vigorous rotational shaking. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. Plasmid DNA was extracted using the QIAfilter Plasmid Midi kit (Qiagen). The procedure involves alkaline lysis of the bacterial cells followed by binding of plasmid DNA to an anion-exchange membrane in the presence of low salt and pH conditions. RNA, proteins and metabolites are removed by a medium salt wash and then the plasmid DNA can be eluted with a high salt buffer. Isopropanol precipitation is used to concentrate and desalt the eluted DNA.

The bacterial pellet was resuspended in 4 ml of buffer P1. 4 ml of buffer P2 was added and the contents of the tube mixed thoroughly by gently inverting 6 times. This lysis reaction was allowed to proceed at room temperature for 5 minutes. 4 ml of chilled buffer P3 was added to the cell lysate and the tube inverted 6 times to precipitate genomic DNA, protein, cell debris and SDS. This mixture was poured into a QIAfilter cartridge. The cartridge was

incubated at room temperature for 10 minutes during which time the precipitated material formed a layer floating on top of the solution. A QIAGEN-tip 100 was equilibrated by adding 4 ml of buffer QBT and allowing it to flow through by gravity flow. The cap was removed from the nozzle of the QIAfilter and the plunger gently inserted into the cartridge. The cell lysate was filtered into the QIAGEN-tip leaving the genomic DNA, protein, cellular debris and SDS in the cartridge which was discarded. The filtered lysate was allowed to pass through the resin of the QIAGEN-tip by gravity flow. The QIAGEN-tip was washed twice with 10 ml of buffer QC before the DNA was eluted with 5 ml of buffer QF. DNA was precipitated by the addition of 3.5 ml of room temperature isopropanol. The contents of the tube were mixed by inversion before being centrifuged at 15000 x g for 30 minutes at 4°C to pellet the DNA. The supernatant was discarded. The pellet was washed with 2 ml of room temperature 70% (v/v) ethanol before centrifugation at 15000 x g for 10 minutes. The ethanol was discarded and the pellet air-dried. The pellet was dissolved in 500 μl of nuclease free water (Promega).

2.3 Eukaryotic cell culture

2.3.1 General tissue culture technique

All tissue culture work was carried out in a class 2 hood. The interior of the hood was decontaminated using 5% (v/v) Decon (Fisher) prior to use. All boxes of pipettes or tips were sterilised by autoclaving. The surface of any pieces of equipment (e.g. pipettes or tip boxes) required in the hood, were decontaminated with 70% (v/v) ethanol before being placed in the hood. Latex gloves, sterilised with 70% (v/v) ethanol, were worn for any work within the hood.

Culturing HIV *in vitro* can lead to concentrations of virus far higher than those found in an infected individual. Thus such work could pose additional infection risks. Any work involving culturing of HIV was carried out in class 2 hood in a category 3 containment lab. While working in this lab, two pairs of latex gloves, plastic sleeve protectors, a plastic apron and a lab coat were always worn. Any waste from this lab was initially decontaminated using 1% (w/v) virkon (Antec), then autoclaved at 121°C for 30 minutes and finally incinerated.

2.3.2 Reagents

Cell lines were grown in either Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) or RPMI media (Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma) and 2 mM L-glutamine (Sigma). Media were supplemented with Geneticin (G418), Hygromycin or Puromycin (Life Technologies) as indicated. Adherent cell lines were harvested using Trypsin (Invitrogen).

2.3.3 Cell lines

Cell line	Species/ Cell type	Growth media	Phenotype notes	References
293T	Human embryonic kidney	DMEM	Weakly adherent monolayer	(Pear et al., 1993)
PM1	Clonal derivative of human cutaneous T-cell lymphoma	RPMI	Grow in suspension	(Lusso et al., 1995)
C8166	Human T-lymphoblastoma	RPMI	Grow in suspension	(Salahuddin et al., 1983);
U373MG	Human glioblastoma/ astrocytoma	DMEM	Adherent monolayer Expresses GFAP	(Ponten and Macintyre, 1968)
A172	Human glioblastoma	DMEM	Adherent monolayer Expresses GFAP	(Giard et al., 1973)
J774	Mouse macrophage	DMEM	Adherent monolayer	(Ralph et al., 1975)
Ghost Indicator Cells CD4	Human osteosarcoma	DMEM + 500 μg/ml Gentomycin G418 + 100 μg/ml Hygromycin	Adherent monolayer Express CD4	(Kewalramani et al., 1998)
Ghost Indicator Cells CD4 CCR5		DMEM + 500 μg/ml Gentomycin G418 + 100 μg/ml Hygromycin + 1 mg/ml Puromycin	Adherent monolayer Express CD4 and CCR5	

Ghost Indicator Cells CD4 CXCR4		DMEM + 500 μg/ml Gentomycin G418 + 100 μg/ml Hygromycin + 1 mg/ml Puromycin	Adherent monolayer Express CD4 and CXCR4	
U87 CD4	Human glioma	DMEM + 300 μg/ml Geneticin G418	Adherent monolayer Express CD4	(Ponten and Macintyre, 1968; Deng et al., 1996)
U87 CD4 CCR5		DMEM + 300 μg/ml Geneticin G418 + 1 mg/ml Puromycin	Adherent monolayer Express CD4 and CCR5	
U87 CD4 CXCR4		DMEM + 1 mg/ml Geneticin G418+ 1 mg/ml Puromycin	Adherent monolayer Express CD4 and CXCR4	

Table 2.3: Cell lines used for HIV production and growth

2.3.4 Freezing cells

Cells were pelleted by centrifugation at 500 x g for 5 minutes. Cells were resuspended at a concentration of 10^6 to 10^7 cells per ml in FCS containing 10% (w/v) dimethyl sulphoxide (Merck). 1ml aliquots of cell suspension were transferred to cryovials (Nunc). These tubes were placed in a freezing box containing isopropanol which was then placed in a freezer at -70ºC. This allows the cells to cool at a rate of approximately 1ºC per minute. After at least 24 hours, the vials were transferred to the liquid nitrogen storage facility.

2.3.5 Thawing cells

An aliquot of frozen cells was removed from the liquid nitrogen storage facility

and thawed rapidly in a water bath at 37°C. 10 ml of media (pre-warmed to 37°C) was added drop wise to the cells in a universal container. The cells were pelleted by centrifugation at 500 x g for 5 minutes. The supernatant was discarded and the cells resuspended in 10 ml fresh media. The cell suspension was transferred to a 25 cm^3 tissue culture flask and incubated at 37°C.

2.3.6 Culturing adherent cell lines

Cells were cultured in 25 cm^3 flasks at 37°C in an atmosphere containing 7% CO_2. Cells were monitored and split when approximately 80% confluence was achieved (every two or three days). To split cells, the media was first discarded. The monolayer was washed with 5 ml sterile phosphate buffered saline (PBS: 7.02 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.137 M NaCl, and 2.68 mM KCl). 1ml 0.05 % (w/v) Trypsin was added and the cells incubated at 37°C until cells detached from the plastic. Cells were resuspended in 10 ml fresh media and 2 mls transferred to a fresh flask. A further 8 ml of fresh media was added and the cells mixed by pipetting.

2.3.7 Culturing cells in suspension

Cells were cultured at in 25 cm^3 flasks at 37°C in an atmosphere containing 5% CO_2. Cells were split every two to three days. The cell suspension was first centrifuged at 500 x g for 5 minutes to pellet the cells. The supernatant was discarded and the cells resuspended in 10 ml fresh media. Cells were counted using a haemocytometer. A portion of the cell suspension was diluted with fresh media to give 10 ml of cell suspension at an approximate concentration of 3 x 10^5 cells/ml. This was transferred to a fresh flask.

2.3.8 Transfection by electroporation

293T cells were suspended in serum free media at a concentration of 10^6 cells/ml. 800μl of cell suspension was mixed with the DNA for transfection. This mixture was transferred to a sterile 0.4 cm electrode gap Gene Pulser Cuvette (Biorad). The cuvette was placed in an electroporator (Biorad, Gene Pulser) and the cell/DNA mixture was subjected to a single pulse of 250 V and 960 μFD capacitance. The cuvette was removed and the cells immediately transferred to a universal tube containing 5 ml of pre-warmed media (containing 10% (v/v) foetal calf serum). Cells were cultured in a small tissue culture flask for 2 days before being analysed.

2.3.9 Liposome transfection

Liposomes are lipid bilayers which form colloidal particles in aqueous medium. The lipid reagent used for transfection was Transfast™ Transfection Reagent (Promega). This reagent consists of a synthetic cationic lipid along with a neutral lipid which has been demonstrated to enhance DNA uptake by cells. The reagent binds DNA and enhances its uptake by cells. The precise mechanism of DNA uptake is unclear but may involve endocytosis or membrane fusion.

The day before a transfection was to be carried out, the vial of transfast reagent was warmed to room temperature and suspended in 400 μl of nuclease free water (Promega). The reagent was then frozen for at least 24 hours before use. Immediately prior to use, the reagent was thawed and mixed by vortexing.

293T cells for transfection were plated the day prior to a transfection being

carried out. Cells were split as described in section 2.3.6 and the number of cells indicated in table 2.4 placed in the appropriate culture container with sufficient media for growth.

Culture container	Approximate growth area (cm^2)	Approximate number of cells to plate	Final volume of media mix (ml)
24 well plate	2	5×10^4	0.2
12 well plate	4	1×10^5	0.4
6 well plate	10	2.5×10^5	1
P25 tissue culture flask	30	7.5×10^5	3

Table 2.4: Areas of culture plates for cell growth

The concentration of the DNA to be used for transfection was assessed using a spectrophotometer (section 2.1.9). In a sterile tube, the DNA was mixed with 15 μl of Transfast reagent per μg of DNA and serum free media to a final volume as indicated in table 2.4. The contents of the tube were mixed thoroughly by vortexing and then incubated at room temperature for 15 minutes. The cells to be transfected were removed from the incubator and the media was removed by pipetting. The DNA/Transfast reagent/media mixture was mixed again by vortexing before it was carefully pipetted onto the cell monolayer. The cells were returned to the incubator for 1 hour. The cells were then overlaid with pre-warmed media containing 10% (v/v) foetal calf serum before being returned to the incubator. Cells were cultured for at least 24 hours before being analysed.

2.3.10 Fluorescent microscopy

An inverted fluorescent microscope was used to observe transfected or

infected cells for expression of the EGFP gene. EGFP was excited at a wavelength of 488 nm and emitted light at 507 nm. A Hamamatsu digital camera was used to take pictures of cells. Pictures were edited using the Openlab computer package.

2.3.11 Harvesting and storing virus

A single cell suspension was formed by pipetting cells. Cells were transferred to a universal container and pelleted by centrifugation at 500 x g for 5 minutes. The supernatant was filtered through a 0.8 μm filter to remove remaining cells and cell debris.

To freeze, 1 ml aliquots of the filtered supernatant were transferred into 2 ml cryovials (Nunc). Vials were carefully labelled before being transferred for storage at -70°C.

When virus was required the vial was removed from the freezer and thawed quickly in water pre-warmed to 37°C.

2.3.12 Infecting PMI cells

Transfected 293T cells were monitored by visualisation using a fluorescent microscope. When a transfection had been successful, green cells were usually visible after two days. In these cases, approximately half of the media was removed from the container and replaced with an equal volume of PM1 cells in suspension in RPMI media at a concentration of approximately 2.5 x 10^5 cells/ml. Cells were co-cultured for up to a week and the non-adherent PM1 cells monitored for signs of infection. In cases where infectious virus was not present there was very little fluorescence visible at this point. Once

the PM1 cells appeared to be infected, approximately half the volume of media was removed from the container and transferred to a fresh container containing an equal volume of PM1 cells at a concentration of approximately 2.5 x 10^5 cells/ml. Cells were monitored daily for infection and additional media added as required.

2.3.13 Infecting indicator cell lines

Virus was harvested from PM1 cells and filtered as described above. 1 ml of virus was added to a well containing 50% confluent cells. The cells were cultured at 37°C and monitored daily for infection.

2.4 Analysis of human and animal tissue

2.4.1 Biological safety when working with infectious material

HIV is classified as a biological pathogen of hazard group 3. Many of the HIV positive samples used are also infected with Hepatitis C. Hepatitis C is also classed as a biological pathogen of hazard group 3. Both these are blood borne viruses and thus the main risk of infection is via a cut with a contaminated sharp implement such as a scalpel, scissors or needle. In addition there is a risk of infection from splashes of infectious material onto the skin or face where virus could enter via a mucus membrane or an existing cut. To minimise such risks all work with infectious material was carried out in a class 2 microbiological safety hood. Two pairs of latex gloves were worn at all times. The use of sharp objects was avoided wherever possible. Laboratory surfaces were regularly cleaned with a solution of 5% (v/v) Decon (Fisher). Any equipment used in the hood was decontaminated with 70% (v/v) ethanol prior to removal. All waste material generated was first decontaminated chemically using 1% (w/v) virkon (Antec) and then autoclaved.

2.4.2 Preparation of fresh brain tissue

Mice were killed by CO_2 inhalation. The aorta was cut to prevent excess blood contamination. The fur on the head was soaked in 70% (v/v) ethanol prior to removal of the brain. The intact brain was rolled on a piece of filter paper to remove meninges. A dissecting microscope was used to visualise the brain and larger blood vessels adherent to the brain surface removed using forceps.

Human brain tissue was supplied in pieces of approximately $1cm^3$ suspended in PBS. Between 15 and 20 of such pieces were supplied for each autopsy. Material included both grey and white matter. The tissue was washed with PBS to remove any excess blood and any visible blood vessels were dissected out.

2.4.3 Collagenase and papain digestion

This method was based on a previously published protocol (Wright et al., 1997). A portion of brain tissue was diced finely using a razor (mice) or forced through a sterile metal sieve (human). Material was suspended in approximately 3 ml Hanks balanced salt solution (HBSS) (Sigma) per 1 ml pulverised brain matter. Crude collagenase (Sigma) was added to a final concentration of 0.1% (w/v) and papain (Sigma) was added to a final concentration of 6 units/ml. Cells were incubated at 37°C for 2 hours with gentle agitation every 20 minutes. Cells were triturated using a Pasteur pipette until no lumps of tissue were visible.

2.4.4 Controlled trypsin digestion

This method was based on a previously published method which was designed to obtain a single cell suspension of brain cells with preserved cellular morphology (Chatterjee and Sarkar, 1984). Brain tissue was cut into a limited number of pieces (each piece was approximately 1 g). Pieces of tissue were added to a volume of digestion medium (3% (w/v) glucose, 3% (w/v) fructose, 0.6% (w/v) albumin, 6mM KH_2PO_4:NaOH and 40% (v/v) trypsin; pH6) at least 4 times the volume of tissue. Brain tissue was incubated at 37°C for 10 minutes with agitation every minute. Buffered foetal

calf serum (FCS) was added (1 ml FCS per ml digestion media used). All further steps were carried out on ice. Tissue was passed through a pipette with a 3 mm opening 20 times and then allowed to settle. The supernatant was removed and stored on ice. Further media (5% (w/v) glucose, 5% (w/v) fructose, 16% (w/v) albumin, 6mM KH_2PO_4:NaOH; pH6) was added (1ml per ml digestion buffer used). Tissue was passed through a pipette with a 2.5 mm opening 20 times and then allowed to settle. The supernatant was removed and pooled with the supernatant previously obtained. Trituration through pipettes of decreasing bore size was continued in this manner until all pieces of tissue had disintegrated.

2.4.5 Standard trypsin digestion

Trypsin was added to a final concentration of 10% (v/v) and the mixture shaken vigorously. Digestion was allowed to proceed for 15-30 minutes at 37°C. 10% (v/v) FCS was added to quench the trypsin activity. Material was triturated through pipettes of decreasing bore diameter until no lumps of tissue were visible. If necessary, extra PBS was added to aid this process.

2.4.6 Liberase and DNAse digestion

This method was based on a previously published method which was used prior to FACS analysis of murine cells (Ford et al., 1995). However, in an attempt to optimise cell yield, crude collagenase was replaced with a purified blend of collagenase and neutral proteases, Liberase blendzyme 3 (Roche). This blend contains less endotoxin than standard preparations of collagenase and has been shown to improve yields of cells obtained from tissues (Linetsky et al., 1997).

A portion of brain tissue was diced finely using a razor (mice) or forced through a sterile metal sieve (human). Tissue was re-suspended in a volume of PBS approximately 4 times that of the volume of tissue. 0.15 units of Liberase 3 (Roche) and 2000 units of DNase (Sigma) were added per ml of PBS. Tissue was incubated at 37°C for 1 hour. During this period, tissue was re-suspended in media using a cut-off 200-1000 µl pipette every 15 minutes. Tissue was triturated through pipettes of decreasing bore size to disaggregate cells.

2.4.7 Obtaining single cell suspension

Suspension was filtered first through a 500 µm filter and then a 200 µm filter (BD Biosciences) to remove any remaining clumps of cells or portions of blood vessels. Suspension was centrifuged at 500 x g for 10 minutes to pellet brain material. Cells were then washed with media (PBS, DMEM or HBBS) to remove any traces of enzymes.

2.4.8 Enrichment of neuron and astrocytes using Ficoll

Pelleted cells were suspended in 50 ml of Ficoll media (9% (w/v) Ficoll (Amersham Biosciences), 8% (w/v) glucose, 5% (w/v) fructose and 10 mM KH_2PO_4:NaOH). The cell suspension was centrifuged at 170 x g for 10 minutes to obtain a neuron enriched pellet. The supernatant was re-centrifuged at 430 x g for 10 minutes. The pellet was discarded. The supernatant was re-centrifuged at 670 x g for 15 minutes to produce an astrocyte enriched pellet.

2.4.9 Separation of cells using a continuous Percoll gradient

Percoll (Amersham Biosciences) was diluted to the desired concentration

using a volume of 10 x PBS equal to 10% (v/v) of the final volume and distilled water. 50 mls of diluted percoll was placed in a tube. A continuous gradient was formed by centrifuging the tube at 30000 x g for 15 minutes. The cells to be separated were suspended in 5 ml of PBS and carefully layed on top of the gradient. The tube was centrifuged at 800 x g for 15 minutes.

2.4.10 Separation of cells using a discontinuous Percoll gradient

Dilutions of Percoll were prepared as above using 10 x PBS and distilled water. Layers of decreasing density were carefully added to a tube in such a way that the interfaces between layers remained distinct. Cells were resuspended in PBS and layered on top. Material was spun at 1250 x g for 25 minutes to separate myelin fragments from cells. After centrifugation a thick cream coloured band of myelin was visible near the top of the percoll while a faint band of cells was visible towards the bottom. The myelin layer was discarded and the cells aspirated. Cells were washed in PBS.

2.4.11 Immunomagnetic separation of cells using the MACS system

All components of the MACS system were obtained from Miltenyi Biotech. Pelleted cells were resuspended in automacs buffer (AMB; PBS supplemented with 0.5% (w/v) bovine serum albumin, 2 mM EDTA- pH7.2) at a concentration of 10^7 cells/80 µl. 20 µl (2 µg) of primary antibody was added per 10^7 cells and the cells incubated at 4°C for 20 minutes. Cells were re-suspended in 20 ml AMB and then centrifuged at 500 x g for 10 minutes. Pelleted cells were re-suspended at a concentration of 10^7 cells/80 µl in AMB. 20 µl Goat anti-mouse IgG MACS beads suspension was added and the

cells incubated at 4°C for 20 minutes. Cells were re-suspended in 20 ml AMB and then centrifuged at 500 x g for 10 minutes. Cells were resuspended in 1 ml AMB. Cells were passed through the AutoMacs machine using the positive selection programme.

2.4.12 Paraformaldehyde Fixation

Paraformaldehyde was dissolved in PBS to the desired concentration (1-4% w/v) in a fume cupboard at a temperature of 50°C. Pelleted cells were re-suspended in paraformaldehyde solution and incubated at 4°C for at least 15 minutes. Cells were stored in paraformaldehyde solution for up to one week. Paraformaldehyde solution was used within three days of preparation.

2.4.13 Triton permeabilisation

Cells were first fixed in paraformaldehyde as described. Cells were pelleted by centrifugation at 500 x g for 10 minutes. The pellet was re-suspended in Triton X-100 (Union Carbide) permeabilisation buffer (0.1% (v/v) Triton X-100, 150 mM NaCl, 1 mM HEPES, 4% (v/v) FCS in PBS). Cells were incubated at 4°C for 15 minutes. Cells were washed twice using PBS.

2.4.14 Gentle Ethanol Fixation

Cells were resuspended in a solution of 50% (v/v) FCS in PBS. The volume used varied between 300µl and 2ml depending on quantity of cells. A volume of 70% (v/v) ethanol four times the volume of 50% (v/v) FCS used was pre-chilled to -20°C. The cells were pipetted up and down vigorously while the chilled 70% (v/v) ethanol was added drop by drop. This prevented clumping of cells. Cells were incubated for at least 2 hours at 4°C. Cells were washed twice with PBS.

2.4.15 Flow cytometric analysis

Antibodies

Antigen	Function	Isotype	Species	Conc. (mg/ml)	Quantity used per million cells (μl)	Conjugate	Supplier
GFAP	Stain astrocytes	IgG1	Mouse	0.4	10	None	Chemicon
Mouse IgG1	As secondary antibody with anti-GFAP antibody	IgG1	Rat	1	5	FITC	Sertec
GFAP	Stain astrocytes	IgG1	Mouse	1	5	Cy3	Sigma
N/A	As isotype control for anti-GFAP Cy3	IgG1	Mouse	0.2	25	Rhodamine (TRITC)	Rockland Inc.
GFAP	To stain astrocytes	IgG1	Mouse	1	5	Alexafluor® 488	Molecular Probes
CD11b	To stain microglia	IgG1	Mouse	0.2	10	FITC	Sigma
CD68	To stain microglia	IgG1	Mouse	0.1	20	FITC	Serotec
N/A	As isotype control for anti-CD11b FITC and anti-CD68 FITC	IgG1	Mouse	0.1	20	FITC	Serotec

Table 2.5: Antibodies used for FACS staining

Cell staining

Before staining cells were washed with PBS to remove fixative if pre-fixed. Cells were suspended in 10% (v/v) normal mouse serum (NMS) (Dako) in PBS and incubated at 4°C for 30 minutes. Cells were pelleted and supernatant mostly removed. Cells were resuspended in drop of supernatant of approximately the same volume as the cell pellet. Primary antibody was added (the volume used is indicated in table 2.5) and the cells mixed by flicking tube. Cells were incubated at 4°C for 1 hour. If primary antibody was conjugated to a flourochrome the tube was wrapped in tin foil during this

incubation. If a secondary antibody was required cells were washed with PBS, pelleted and incubated with antibody as previously. Cells were washed twice with PBS. Cells were either fixed or, if previously fixed, resuspended in PBS.

2.4.16 Preparing cytospins

All cytospin products were obtained from Thermo Shandon. A wash slide and filter card were assembled in the cytoclip which was secured in the cytospin machine. Initially 50 µl of PBS buffer was added to the well and the slides spun at 150 rpm for 3 minutes. These slides were discarded and replaced with clean labelled slides. 50 µl of cell suspension (at a concentration of 1-5 x 10^5 cells/ml) was added to the well. Slides were spun at 150 rpm for 3 minutes. Slides were removed and air-dried.

2.4.17 GFAP Immunocytochemistry

Slides were fixed in acetone/methanol for 20 minutes. Cells were blocked in 100 ml methanol + 25 ml H_2O_2 and then washed with H_2O. Slides were inserted into Sequenza Immunostaining center (Thermo Shandon) for all further steps. Slides were washed twice with PBS before being incubated in 20% (v/v) normal swine serum for 10 minutes. 100 µl of primary polyclonal anti-GFAP antibody (Dako) (diluted 1:1500) was added and slides incubated for 30 minutes. Slides were washed twice in PBS before 100 µl of biotinylated swine anti-rabbit IgG antibody (Dako) (diluted 1:200) was added and the slides incubated for 30 minutes. Stained cells were visualised using the StrepABC kit and DAB solution (DAKO) according to the manufacturer's instructions. Slides were washed with H_2O before counterstaining with haematoxylin. Slides were dehydrated, cleared and mounted.

2.4.18 Phenol/Chloroform extraction

500 µl lysis buffer (0.11 M $NaCl_2$, 55 mM Tris pH 8.0, 1.1 mM ethylenediamine tetraacetic acid (EDTA) pH 8.0, 0.55% (w/v) sodium dodecyl sulphate (SDS), 1 mg/ml Proteinase K, 40 µg/ml poly A) was added to a block of tissue of approximately $1cm^3$ or a cell pellet of FACS sorted cells in a 1.5 ml eppendorf tube. Tube was mixed by vortexing and incubated at 65°C for two hours or until tissue was completely dissolved. 450 µl of H_20 saturated phenol (Sigma) was added and the tube vortexed for 5 minutes. The mixture was centrifuged at 12000 x g for 10 minutes at room temperature. The aqueous layer was transferred to a new eppendorf tube containing 450 µl chloroform/isoamylalcohol (Rathburn) 50:1 and mixed by vortexing for two minutes. The mixture was again centrifuged at 12000 x g for 10 minutes at room temperature. The aqueous layer was transferred to a new eppendorf tube containing 40 µl 3 M sodium acetate (NaAc) pH 5.2 and 800 µl 100% ethanol (pre-chilled to -20°C). The tube was mixed by inversion and then incubated overnight at -20°C to allow precipitation of nucleic acids. Nucleic acid was precipitated by centrifugation at 12000 x g for 10 minutes at 0°C. The pellet was washed with 80% (v/v) ethanol and then centrifuged at 12000 x g for 10 minutes at 0°C. The supernatant was removed and the pellet dried on a hot block at 37°C for 10 minutes. The pellet was resuspended in 50 µl nuclease free water (Promega).

2.4.19 Extraction using Qiagen DNeasy® Tissue kit

If DNA was to be extracted from cells in suspension (i.e. After FACS sorting), the cells were pelleted by centrifugation in a 1.5 ml eppendorf tube and the

supernatant discarded. If a frozen piece of tissue was to be used for DNA extraction, approximately 25 mg of tissue was excised and cut into small pieces before being added to a 1.5 ml eppendorf tube.

180 µl of buffer ATL and 20 µl proteinase K were added. Contents of tube were mixed thoroughly by vortexing before being placed in a hot block to incubate at 55°C. Digestion was allowed to proceed for between 4 and 16 hours with occasional mixing by vortexing until all aggregates of tissue had dispersed. Following incubation, tube was vortexed for at least 15 seconds to ensure complete mixing of contents. 200 µl of buffer AL were added and the contents of tube mixed thoroughly by vortexing. Tube was incubated at 70°C for 10 minutes. 200 µl of 100% ethanol was added and the contents of the tube mixed thoroughly by vortexing.

The mixture was pipetted into a DNeasy Mini Spin Column placed in a 2 ml collection tube. The tube was centrifuged at 6000 x g for 1 minute allowing the mixture to pass through the silica membrane of the column. DNA becomes bound to the silica membrane in the presence of high salt concentrations and is thus retained. The DNeasy Mini Spin Column was transferred to a clean collection tube. Two wash steps are carried out to remove any contaminants which may be present. First 500 µl of buffer AW1 was added and the tube centrifuged at 6000 x g for 1 minute. Secondly 500 µl of buffer AW2 was added to the column. In this case centrifugation at 6000 x g was carried out for 3 minutes in order to completely dry the silica membrane. The column was once again transferred - to a clean 1.5 ml

eppendorf tube.

200 μl of nuclease free water (Promega) was added to the centre of the silica membrane. The tube was incubated at room temperature for 1 minute before being centrifuged at 6000 x g for 1 minute. DNA is released from the silica membrane in the presence of low salt concentrations and thus is eluted in the water.

Chapter 3: Results I

3.1 *Introduction*

HIV particles are encapsulated by a host-derived lipid membrane. Embedded in this membrane are complexes of Env proteins (reviewed in Berger et al., 1999; Dragic, 2001). Each complex consists of three transmembrane gp41 portions non-covalently attached to three surface gp120 portions. It is thought that binding of gp120 to host cell CD4 causes a conformational change which then allows it to bind its co-receptor. This causes a second conformational change which allowing gp41 to fuse with the host cell membrane and release the contents of the virion into the cell. As Env mediates both cell binding and membrane fusion, it is the major determinant of the cellular tropism of a given HIV particle. The *env* gene is the most variable in the HIV genome and this contributes to the differing abilities of HIV variants to infect different cell types. One important factor in the cellular tropism of HIV is that different variants can use different co-receptors. Primary HIV-1 virions most commonly use the chemokine receptors CCR5 and CXCR4 as a co-receptor. Virions can use only CCR5 (R5 variants), only CXCR4 (X4 variants) or either (dual-tropic variants). Some associations between co-receptor usage and genetic factors have been noted. For example, there is an association between the number of basic residues in the V3 loop of gp120 and the ability to use CXCR4 (Fouchier et al., 19992; Basmaciogullari et al., 2002). In particular, the presence of positive charged amino acids at amino acid positions 324 and 335 of *env* is strongly associated with CXCR4 usage. Substitutions at a number of amino acid sites in gp120 are correlated with a switch from CXCR4 to CCR5 usage *in vivo* (Rizzuto et al., 1998). In some cases, the

ability of HIV variants to infect certain cell types is associated solely with its ability to utilize a certain co-receptor. However, there are also many subtle differences in the cellular tropism of HIV variants which have not been linked to receptor usage alone. For example it is not clear why T-tropic isolates cannot use CXCR4 to infect primary macrophages (Yi et al., 1998). Thus even where the receptor usage of a given Env can be predicted from its sequence, this gives only a partial understanding of the cellular tropism which it would confer to a virion. In order to study subtle variations in tropism it is necessary to use *in vitro* assays to determine the phenotype of different Envs.

It is possible to assess viral tropism by culturing HIV from an infected individual and then testing its ability to infect cells *in vitro*. This method was used in some of the first studies of HIV tropism (McDougal et al., 1985; Klatzmann et al., 1984). The main problem with this approach is that the virus must first be amplified by passage in cell lines. Clearly the selective pressures acting on the viral population *in vitro* are substantially different to those encountered in the human body. The virus initially obtained from the individual will contain an array of genetic variants but passage in cell culture will quickly select those most suited to lab conditions (Vartanian et al., 1991). Perhaps most importantly, the virus will no longer face the challenge of evading the immune system and thus variants which would be swiftly neutralised *in vivo* may become dominant. Any findings regarding the tropism of this virus may not reflect the true *in vivo* situation. A second drawback of this method is that as a large number of genetically distinct HIV variants will be present, it is difficult to note associations between observed phenotypes

and genetic factors. Also, as there will be genetic variations across the whole genome and not just the *env* gene, it is possible that observed differences between variants may reflect differences in infectivity as apposed to true differences in cellular tropism.

In order to circumvent these problems it is necessary to produce separate populations of virus which are genetically identical except in the gene of interest. There are two slightly different methods which are commonly used to achieve this. In both cases recombinant DNA technology is used to amplify HIV genetic material in prokaryotic cells. The DNA is used to transfect a eukaryotic cell line. Once the HIV genome enters the eukaryotic nucleus, the cell begins to act in the same manner as a HIV infected cell and produces HIV particles. Providing the DNA used for transfection of a population of cells is clonal, the virions produced should be almost identical. Only slight variations due to one round of transcription error will exist.

The first of these methods is known as pseudotyping. In this technique, an HIV genome with a defective *env* gene is transfected into the eukaryotic cell line. A plasmid containing the functional *env* gene to be investigated along with regulatory sequences required for expression are used to co-transfect the same cells. Virions will be produced from cells with functional Envs on their surface however the packaged RNA genomes will include the defective *env* gene. These "pseudotyped" viruses can be used to infect cell lines to assess tropism but these cells will not themselves produce infectious virus. Pseudotyped retroviruses have been used in research for many years (Hanafusa, 1965) and so this technique was quickly adapted for use with HIV

after the discovery of the virus. A large amount of research work into tropism continues to be carried out using pseudotyped HIV (Schmitz et al., 2004; Canki et al., 2001).

An alternative method is to produce infectious chimeric virus. Recombinant DNA technology is used to insert the *env* gene of interest into a plasmid which contains the rest of the HIV genome. The *env* gene must be inserted in the correct position in the correct frame. When this construct is transfected into a cell line, infectious virus bearing the Env to be investigated will be produced. In this case, the RNA genome will also possess the *env* gene of interest and thus, providing the *env* gene is functional, infectious virus will be produced. This technique has been used in a number of recent studies investigating tropism (Dittmar et al., 2001; Trouplin et al., 2001).

One obvious advantage of using the pseudotype method is that the virus can only undergo one round of replication and therefore poses less of a threat to human health than infectious virus. On the other hand, every time a pseudotyped virus is required, a transfection must be carried out whereas stocks of infectious chimeric virus can be grown up and stored for multiple experiments.

These methods largely overcome the problem of selection of variants by passage in eukaryotic cells. However, amplification of the DNA in prokaryotic cells can also exert a selective pressure on the genome. Lentiviral genomes often contain cryptic promoters which lead to translation of viral proteins in the prokaryotic cells (Cunningham et al., 1993). Genomes amplified in *E. coli* are often found to be non-functional or truncated presumably because

functional proteins, when expressed, are toxic to the cells (Peden, 1992). Indeed both Env and protease have been found to be toxic when expressed in *E. coli* (Kusumi et al., 1992; Baum et al., 1990). In addition to selecting for pre-existing defective genomes, growth in *E. coli* can result in new mutations occurring within the genome. In particular, it is common for plasmids containing the entire HIV genome to recombine at the viral LTRs thus eliminating the genome (Yamada et al., 1995a). If the viral genome is causing any inhibitory effect then cells containing these "looped-out" plasmids will soon become the dominant variety.

One way to prevent selection of pre-existing defective genomes is to use genetically identical copies of the gene of interest (i.e. derived from a single proviral copy) for every transformation of *E. coli*. If this is carried out then there will be no varieties of plasmid to compete for survival; any clones which are obtained should contain the same gene (although mutation during growth in the *E. coli* can still occur). In order to obtain copies of genes which are derived from a single copy, it is necessary to dilute the template molecules to a concentration such that most PCRs carried out on it will be negative (Simmonds et al., 1990). This technique, known as limiting dilution PCR, ensures that the few reactions which are positive are very likely to have utilised a single copy of template. If a number of alleles of the same gene are obtained, and each used for separate transformations then clones which reflect the diversity *in vivo* can be obtained.

Using limiting dilution PCR to obtain *env* genes necessary for either a pseudotyping or chimeric virus assay would limit the artefactual selection

pressures on the gene outlined above. However, as mentioned, mutations can also occur within *E. coli* and if these confer a selective advantage to cells they may give rise to DNA sequences which are not reflective of those found *in vivo*. In addition, if a given *env* sequence is particularly toxic it may not be possible to amplify it using *E. coli* at all. For these reasons a method which completely eliminates DNA amplification in prokaryotic cells would be highly desirable.

3.2 Aims

The primary aim of this study was to produce a system that could be used to assess the cellular tropism conferred to infectious virus by a given *env* gene. A vector was produced which could be used to generate chimeric HIV genomes containing *env* genes amplified at limiting dilution from patient-derived DNA. The ability of cells transfected with the vector to produce infectious virus was tested.

A secondary aim was to investigate methods of using the vector to produce infectious virus without the need to amplify DNA in prokaryotic cells.

3.3 Results

3.3.1 Producing pNL4.3ΔenvEGFP

In order to produce chimeric virus containing PCR amplified *env* genes, a vector was required which contained the entire HIV genome except the *env* gene. The vector chosen as the basis of this new construct was pBluescript NL4.3 2K (kindly donated by Matthias Dittmar). Two manipulations of the vector were required. First the *env* gene was removed and secondly a reporter gene was added in order that infection with the chimeric virus could

be readily observed and quantified. The final construct also possessed restriction sites allowing the ligation of new *env* genes.

The reporter gene chosen to be inserted into the vector was the enhanced green fluorescence protein (EGFP) a mutant of the *Aequorea victoria* jelly fish green fluorescence protein (GFP) (Cormack et al., 1996). EGFP fluoresces up to 100 times more brightly than GFP when excited at 488nm. This property means that it can be readily detected by fluorescence activated cell sorting (FACS). Including this protein in the vector allows infection by the resultant chimeric viruses to be easily monitored either by FACS analysis or by observing infected cells under a UV microscope.

The strategy devised to carry out the required manipulations is outlined in figure 3.1. The *env* gene was first removed by digesting the vector with *Acc 65* I (an isoschizomer of *Kpn* I). The two *Acc65* I sites which normally exist in the *pol* gene of NL4.3 had been previously eliminated (Dittmar et al., 2001). There are two further *Acc65* I sites in NL4.3. One is 139 base-pairs downstream from the start codon of *env* while the second lies 304 bases after the end of *env* within the open reading frame of *nef*. Thus, digestion with this enzyme results in the excision of the majority of the coding region of *env*. Significantly, cryptic promoters which lead to expression of viral proteins in *E. coli* have been identified in other lentiviruses at the extreme 5' end of the *env* gene (Cunningham et al, 1993). It was hoped that retaining the first 139 bases of the lab adapted NL4.3 *env* sequence might enhance stability of inserted *env* genes when grown in *E. coli*. After restriction digestion, the linearised vector was purified by agarose gel extraction.

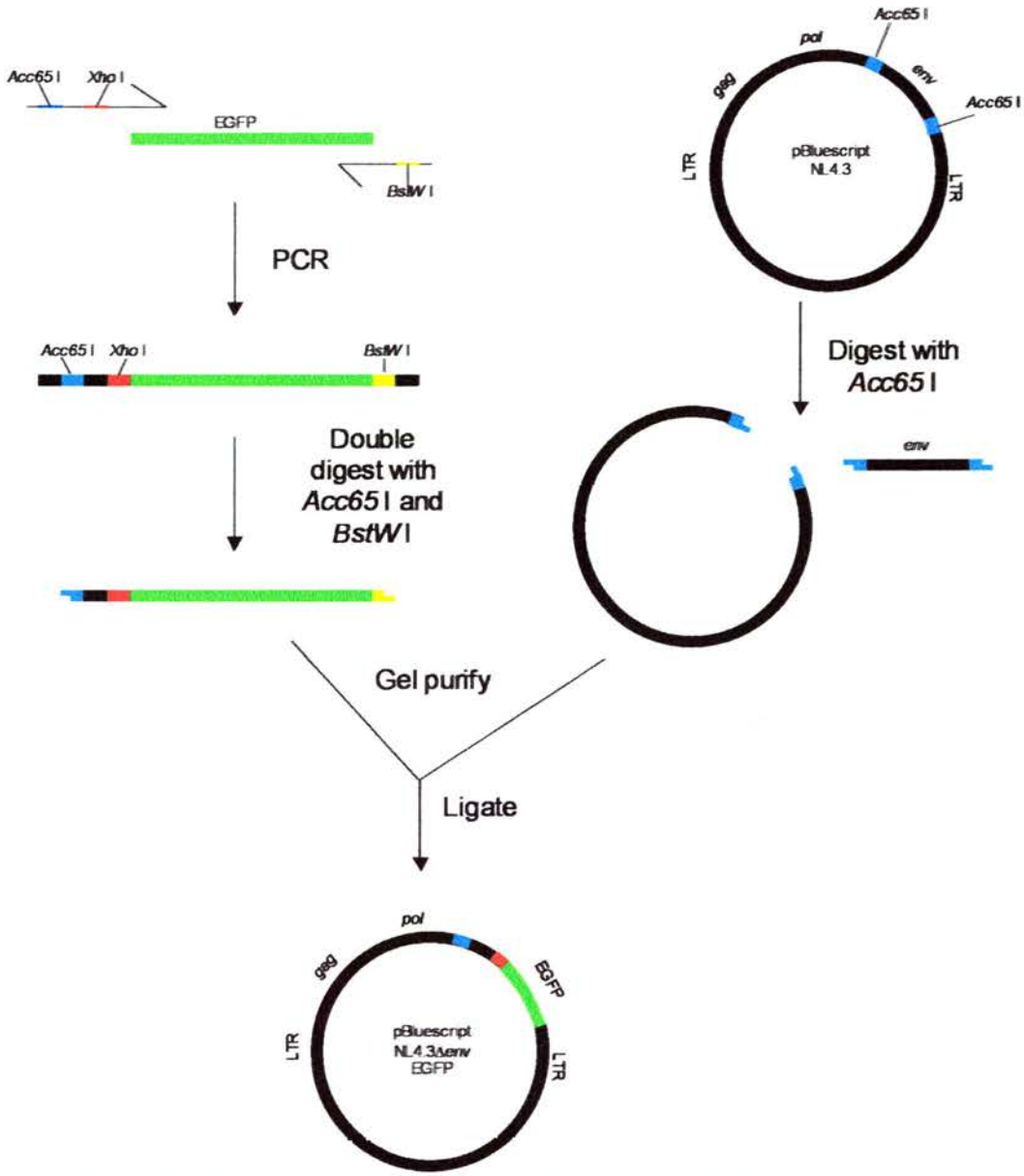


Figure 3.1: Strategy used to produce pBluescript NL4.3Δ*env*EGFP. EGFP was amplified using primers which incorporate restriction sites as shown. This fragment was then digested with the outer restriction enzymes *Acc* 65 I and *BstW* I. The plasmid pBluescriptNL4.3 was digested with *Acc* 65 I. Both the EGFP fragment and the cut vector were gel purified to remove fragments which could interfere with the ligation. The EGFP fragment was ligated into the vector. The overhang left by *BstW* I is compatible with that produced by *Acc* 65 I. The resultant sequence is not cut by either enzyme.

The EGFP gene was inserted into the vector in such a way that when a PCR generated *env* fragment was also inserted, EGFP would be situated in frame with the start of *nef*. To achieve this, for amplification of EGFP, primers that encoded a short fragment of new genetic material to be incorporated at both the 5' and 3' ends of the PCR product were designed. The 5' primer encoded an *Acc65* I restriction site. This meant that when the fragment was digested with *Acc65* I it could be ligated to the left-hand *Acc65* I "sticky" end of the linearised vector. The 5' primer next encoded a short linker sequence before the restriction site for *Xho* I. Most subtype B HIV sequences have a *Xho* I site towards the beginning of the Nef open reading frame (approximately 150 bps after the end of *env*). This is the only *Xho* I site found in most subtype B *env*s. The 5' end of the 3' primer encoded a *BsiW* I restriction site. When DNA is digested with this enzyme a sticky end is generated that is compatible with the sticky end generated by *Acc65* I digestion. Thus, after digestion with *BsiW* I the fragment could be ligated at its 3' end to the right-hand *Acc65* I sticky end in the vector left by the excision of *env*. Ligation of these two ends thereby eliminated this *Acc65* I site. The now unique *Acc65* I site at the 5' end of *env* along with the newly introduced *Xho* I site could be used to ligate in PCR generated and *Acc65* I and *Xho* I digested *env* genes. The primer encoding the *Xho* I site was designed such that when the *env* fragment was ligated into the vector, the beginning of *nef* would be in frame with the EGFP gene. Thus the EGFP gene would be expressed as a Nef fusion protein.

The ligation mixture containing the EGFP fragment and the linearised vector was used to transform *E. coli*. The cells were cultured on ampicillin

containing agar plates at 37°C for 16 hours and resultant colonies used to inoculate 3 ml of LB broth which was incubated at 37°C for 16 hours. Initial minipreps of these cultures revealed a plasmid of approximately 3kb. It is likely that this plasmid had arisen due to homologous recombination of the plasmid at the LTRs leading to the removal of the viral genome as previously reported (Yamada et al., 1995b). Growth of the 3 ml cultures at 30°C eliminated this problem. Figure 3.2 shows a restriction digest of extracted plasmid DNA obtained from individual colonies. The EGFP fragment could potentially ligate into the vector in either direction. Digestion with *Sbf* I and *Kpn* I (both unique restriction sites within this vector) was used to identify a plasmid where the fragment had inserted in the desired orientation.

3.3.2 Production and growth of virus

The first step towards producing virus with the new vector was to insert an *env* gene. Initially the NL4.3 *env* gene was cloned back into the vector. PCR amplification of *env* was carried out using pBluescript NL4.3 as a template. The gene was gel purified and double digested with *Acc65* I and *Xho* I. The vector, pNL4.3Δ*env*EGFP was also double digested with *Acc65* I and *Xho* I and gel purified. A ligation reaction was performed using approximately 3 times the molar concentration of insert compared to vector. Figure 3.3 shows an outline of this strategy. This ligation reaction was used to transform *E. coli* which were then cultured on ampicillin containing agar plates at 37°C for 16 hours. Individual colonies were selected to inoculate 3 ml aliquots of LB broth which were incubated at 30°C for 16 hours. The plasmid DNA was extracted from each of the broth cultures and double digested with *Acc65* I and *Xho* I to

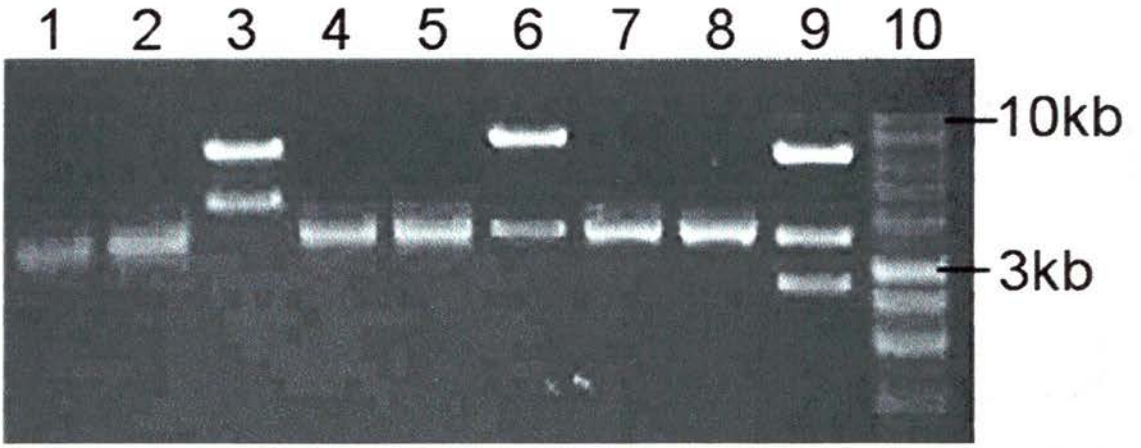


Figure 3.2: Double digests of plasmids with *Sbf* I and *Kpn* I. Lanes 1 to 8: minipreps; Lane 9: pNL4.3; Lane 10 1kb DNA marker. Digestion of intact pNL4.3 results in 3 bands: one at approximately 2.5 kb (*env*), one at approximately 3.5 kb and one at approximately 7 kb as seen in lane 9. The cloning strategy aims to eliminate *env* and incorporates EGFP (0.75 kb). If the EGFP fragment has inserted in the correct orientation, the largest fragment will be approximately 7.75 kb while the second fragment will remain approximately 3.5 kb. This pattern is observed in lane 6. Note that in lane 3 the small fragment is approximately 4.25 kb indicating that in this case the EGFP fragment has inserted in the wrong orientation.

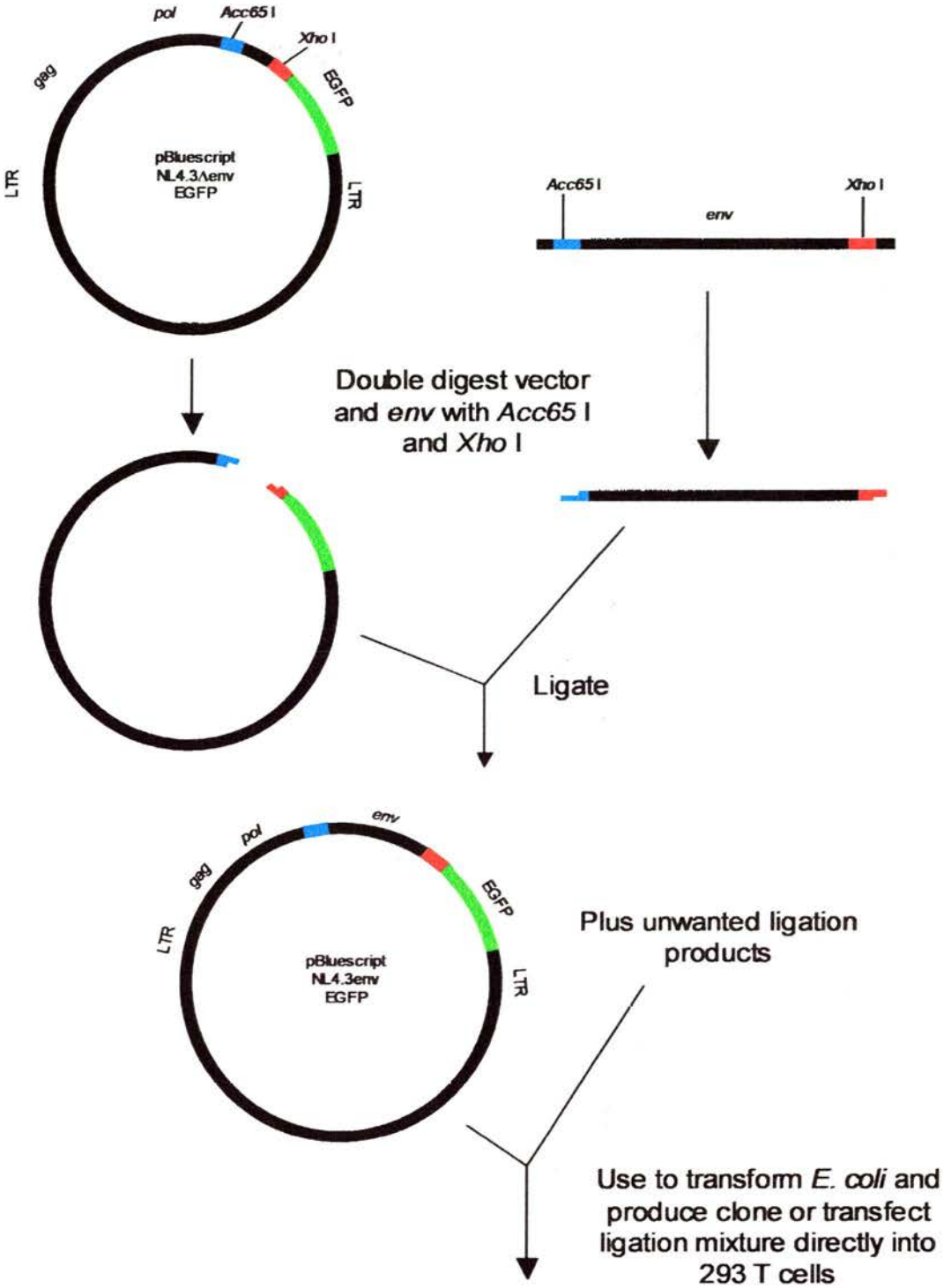


Figure 3.3: Schematic diagram outlining strategy used to insert *env* gene by ligation. PCR is used to amplify *env*. Both *env* and the vector are digested with *Acc* 65 I and *Xho* I. Ligation of *env* into the vector can be carried out. As the overhanging ends of *Acc* 65 I and *Xho* I are not compatible, the fragment can only insert in the correct orientation.

check for the presence of the insert. Figure 3.4 shows that the majority of clones tested did contain the insert. This is as expected because using two restriction enzymes to cut the vector and then gel purifying the linearised vector means that the vector cannot self-ligate; an insert must be present. In addition, the use of different restriction sites at the ends of *env* means that the gene could only insert in the correct orientation.

Vector produced in this manner could be used to transfect mammalian cells. Before a transfection, 293T cells were grown to approximately 80% confluence in a 25 ml tissue culture flask. Approximately 1 µg of plasmid DNA was used to transfect the cells by lipid transfection using Transfast media and the cells were incubated at 37°C in the presence of 5% CO_2. Note that a log of this and all further transfection experiments can be found in appendix 1.

After one day, a faint glow was apparent when cells were observed at 10 times magnification under UV light. By day two, a number of brightly fluorescing cells were visible as shown in figure 3.5. It was estimated that approximately 1 in 50 cells were brightly fluorescent although many more were dimly fluorescent. At this stage, 5 ml of media was removed from the flask and 5 ml of C8166 cell culture (at a concentration of approximately 2.5 x 10^5 cells/ml) was added. After two further days, when a few of the C8166 cells appeared to be infected, 5 ml of media and non-adherent cells were transferred into a flask containing 5 ml of C8166 cell culture at a concentration of approximately 2.5 x 10^5 cells/ml. Within a week many brightly glowing cells could be observed and by week two almost every cell

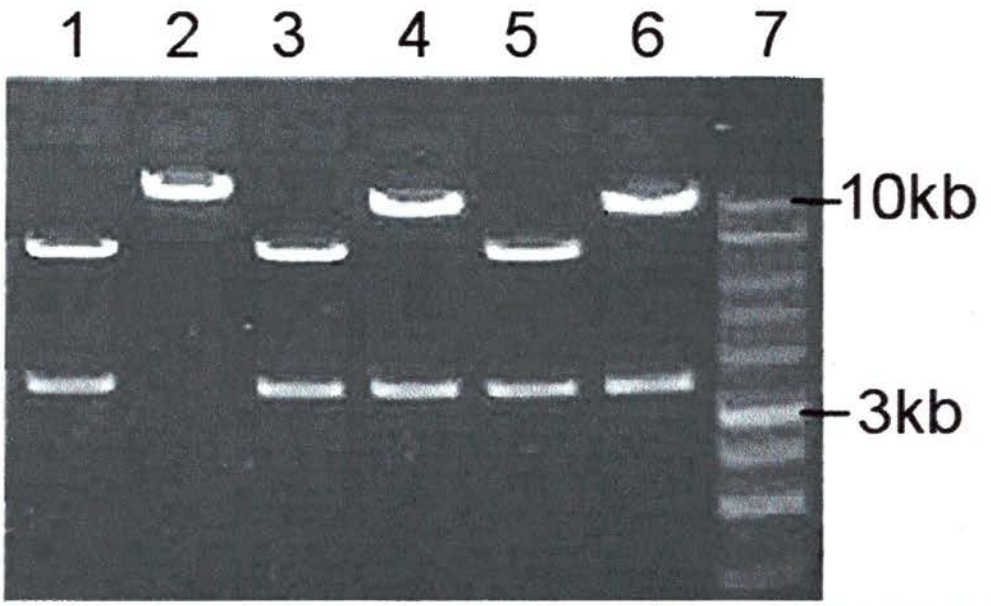


Figure 3.4: Plasmid DNA double digested with *Sbf* I and *Kpn* I. Lanes 1 to 6: minipreps; Lane 7: 1kb marker DNA. If the vector has failed to digest or digested and self-ligated the approximate size of fragments expected would be 7.75 kb and 3.5 kb. This pattern is observed in lanes 3 and 5. If *env* has been successfully inserted, the larger fragment should be approximately 10kb while the small fragment should remain 3.5kb. This pattern is observed in lanes 5 and 6.

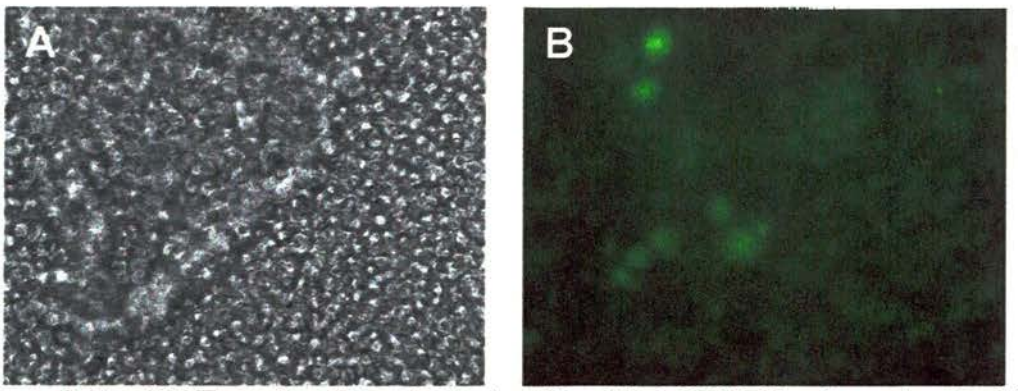


Figure 3.5: 293T cells transfected with NL4.3EGFP viewed at 10X magnification using (A) normal light and (B) UV light after 2 days incubation

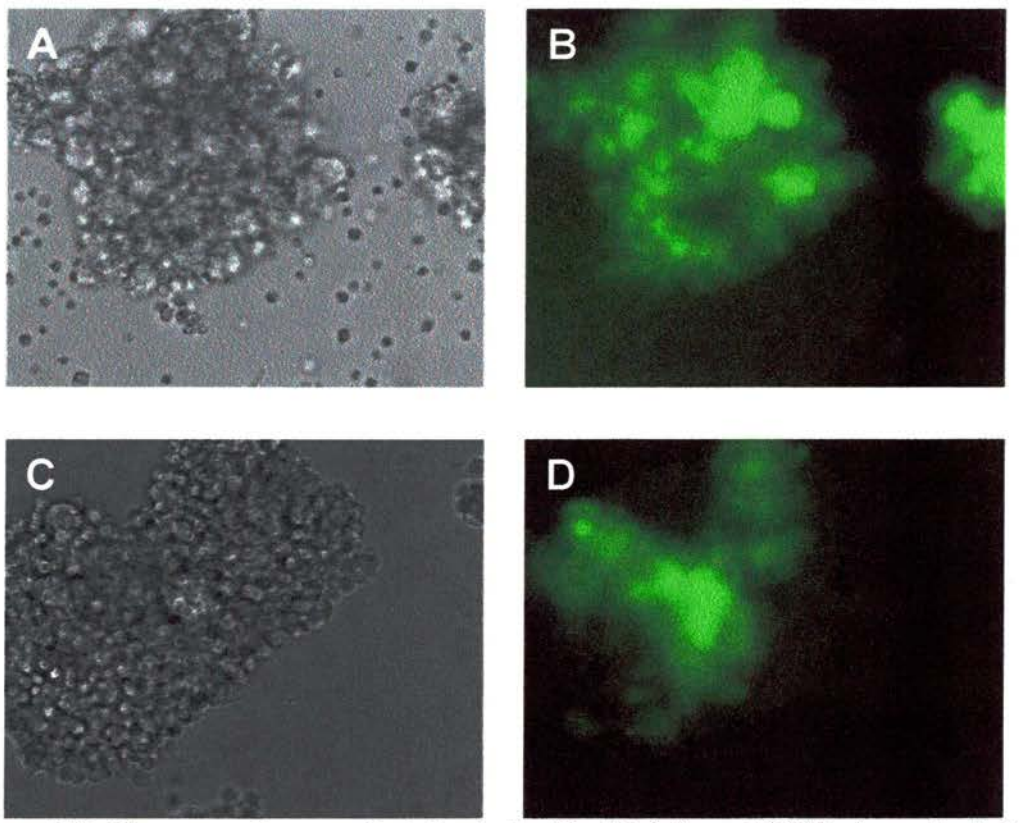


Figure 3.6: Tissue culture cells infected with NL4.3*env*EGFP viewed at 10X magnification. C8166 cells infected with NL4.3*env*EGFP viewed under (A) normal and (B) UV light. PM1 cells infected with NL4.3ADA*env*EGFP under (C) normal and (D) UV light.

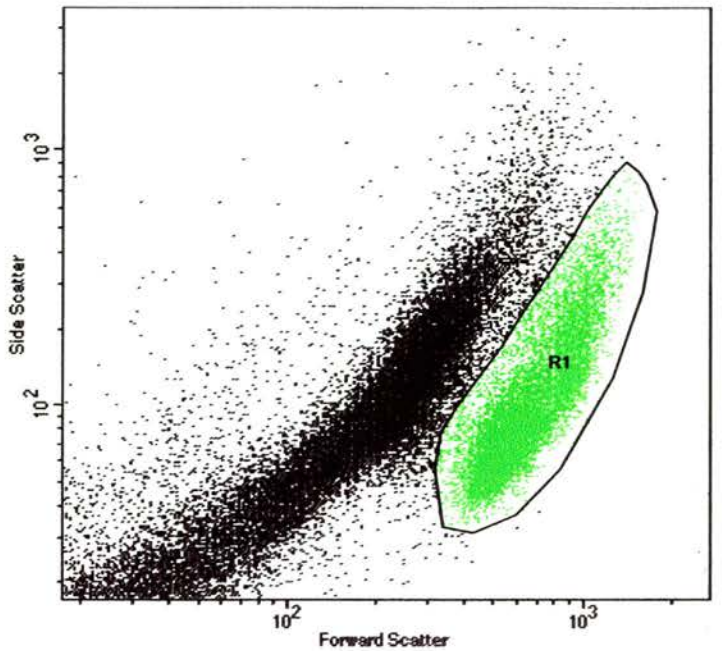
appeared to be infected as seen in figure 3.6 (A/B). Numerous syncytia were also observed.

The method outlined above was repeated to produce virus possessing different *env* genes. Infectious virus possessing the *env* genes from three common lab strains: HXB2, YU2 and ADA were produced. HXB2 is an X4 variant while ADA and YU2 are R5 variants. For each *env* gene, miniprepped plasmid was transfected. Transfection of 293T cells with the plasmids resulted in similar numbers of fluorescing cells as had been observed with transfection of the NL4.3 *env* containing vector. As C8166 cells do not possess the CCR5 chemokine receptor they could not be used for infection with the ADA or YU2 *env* containing virus. Instead, PM1 cells which possess both CCR5 and CXCR4 were used for amplification of all four viruses. Again the PM1s were first co-cultured with the transfected 293T cells before being transferred to new flasks containing fresh cells. The virus containing HXB2 *env* grew in a manner very similar to the virus containing NL4.3 *env*; after one week many cells were infected and by week two most cells appeared infected. Many syncytia were also observed. In contrast, the ADA and YU2 *env* containing viruses grew more slowly. Patches of infection were seen within clumps of cells however even after a number of weeks, there were many more uninfected cells than infected (figure 3.6C/D). This result is consistent with the slow growing phenotype characteristic of R5 viruses. The virus containing YU2 *env* had a particularly slow growing phenotype and thus the ADA *env* containing virus may be more useful for future experiments.

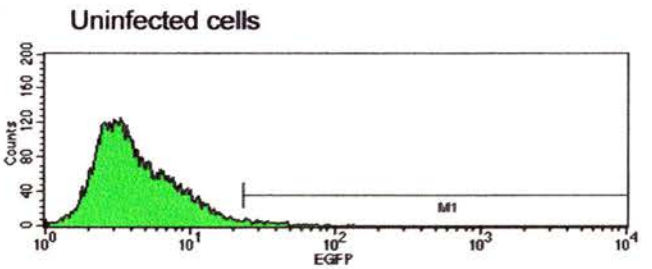
Attempts were also made to produce the viruses possessing the *env* gene from the dual-tropic variants Sf162 and 89.6. Unfortunately, although fluorescence was apparent in 293T cells transfected with clones possessing these *env* genes, no infectious virus was detected. It is unclear why this occurred. Possibly the clone used as a template for PCR amplification of the *env* gene contained a defective copy of this gene. Initial attempts to produce virus with a YU2 *env* gene were also unsuccessful. It later transpired that the clone used as PCR template for the YU2 *env* gene was defective. When an alternative YU2 clone was used as template for the *env* PCR, it was possible to produce infectious virus possessing the YU2 *env* gene.

An alternative explanation is that growth of the plasmids containing the Sf162 and 89.6 *env* genes in *E. coli* could have led to the selection of defective copies of *env* generated by PCR. There is some evidence that this process was occurring with other *env* genes. For example, in the experiment started on 3/02/03 (see Appendix 1 for details), only 2 of the 5 ADA *env* containing clones were capable of producing infectious virus. Since all of these clones used an *env* PCR fragment derived from the same cloned template, it seems likely that selection of defective copies occurred in *E. coli*.

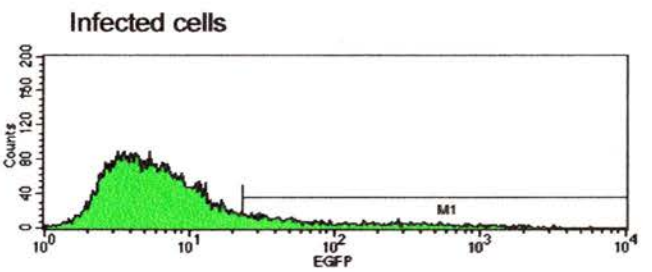
Inclusion of the EGFP gene in the chimeric virus allowed infection to be monitored by FACS. PM1 cells which had been infected for 1 week with NL4.3ADA*env*EGFP were harvested by centrifugation and fixed in 4% (w/v) paraformaldehyde. These cells were analysed using a FACscalibur flow cytometer. The results are shown in figure 3.7. There was a marked increase in fluorescence emitted by infected compared to uninfected cells.



Region	Events	% Total
R1	15229	30.46



Marker	Events	% Gated	% Total
All	15229	100.00	30.46
M1	167	1.10	0.33



Marker	Events	% Gated	% Total
All	15593	100.00	31.19
M1	1772	11.36	3.54

Figure 3.7: Flow cytometric analysis of uninfected and infected PM1 cells. In the histogram plot of uninfected cells it can be seen that 1.10% of cells have sufficient fluorescence to fall within the marker M1. In the histogram plot of infected cells it can be seen that this number rises to 11.36%.

FACS analysis could be a valuable and objective method of monitoring infection in future studies using the chimeric virus technique.

The four viruses (containing NL4.2, HXB2, ADA or YU2 *env* genes) were tested for co-receptor usage. 5 ml of culture medium was removed from PM1 cells which had been infected for 2 weeks. The media was filtered through a 0.22 µm filter. The media was then added to a 25 ml flask containing indicator cells grown to approximately 80% confluence. Table 3.1 summarises the abilities of the four viruses to infect indicator cell lines. Initially ghost cells expressing CD4 in conjunction with either no co-receptor, CCR5 only or CXCR4 only were used. Infection of these cells with the YU2 *env* containing virus is shown in figure 3.8. Surprisingly, syncytia were observed in the CCR5 expressing ghost cells when infected with the YU2 *env* containing virus. Syncytia formation is not usually associated with *in vitro* growth of R5 viruses although this finding is consistent with the observation of syncytia in HIV infected brains (where R5 variants predominate) (Sharer, et al., 1985) and with some other *in vitro* studies (Gorry et al., 2002; Lai et al., 2001). Unfortunately, it was discovered that all ghost cells express CXCR4 at low levels and therefore the co-receptor usage of viruses could not be accurately assessed using this cell line. This explains the finding that the NL4.3 and HXB2 *env*-containing viruses were able to infect all types of ghost cells. U87 cells which express either no co-receptor, CCR5 only or CXCR4 only, in conjunction with CD4, were used for further experiments. In this case, the results obtained were as expected from the predicted phenotype of the *env* genes. This confirmed that the virus possessed the correct *env* genes.

	Ghost cells			U87 cells		
	CCR5	CXCR4	CD4 only	CCR5	CXCR4	CD4 only
NL4.3EGFP	+	+	+	-	+	-
NL4.3HXB2envEGFP	+	+	+	-	+	-
NL4.3ADAenvEGFP	+	-	-	+	-	-
NL4.3YU2envEGFP	+	-	-	+	-	-

Table 3.1: Infection of co-receptor expressing cells by harvested virus incorporating the *env* gene from common lab strains of HIV. +: Infection was observed; –: Virus did not infect the cell line. NL4.3 and HXB2 are CXCR4 using strains while ADA and YU2 are CCR5 using strains. Results are largely as expected given these predicted phenotypes. One surprising result is that both NL4.3EGFP and NL4.3HXB2*env*EGFP are able to infect all Ghost cell lines. The explanation for this is that all varieties of this cell line express low levels of CXCR4.

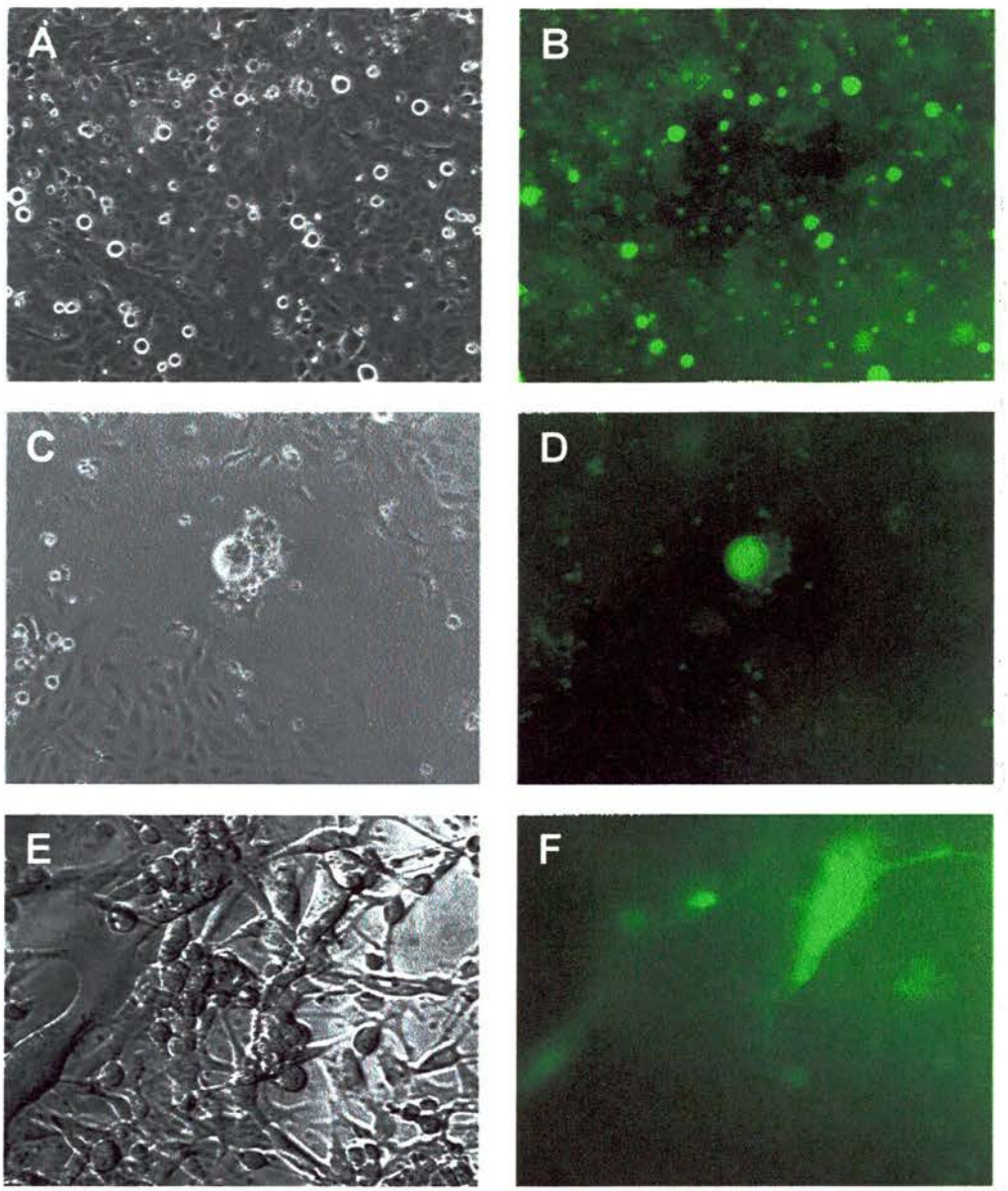


Figure 3.8: Co-receptor expressing cells infected with NL4.3ADA*env*EGFP virus viewed at 10X magnification. CCR5 expressing ghost cells under (A) normal and (B) UV light; Syncytia formation in CCR5 expressing ghost cells under (C) normal and (D) UV light; CCR5 expressing U87 cells under (E) normal and (F) UV light.

The ability of the system to produce infectious viruses containing *env* genes amplified from patient DNA was tested. DNA was extracted from tissue samples obtained from the right occipital and the left temporal lobes of the brain of a study subject who had died of AIDS. Limiting dilution PCR was carried out to obtain ten *env* fragments for each brain region. These *env* fragments were cloned into pNL4.3Δ*env*EGFP as described previously. DNA extraction, limiting dilution PCR and cloning were carried out by Mathew Marsden, a post-doctoral fellow working in the group (Marsden et al., In press). The resultant clones were used to transfect 293T cells. PM1 cells were co-cultured with the 293T cells for two days before being transferred to a flask containing fresh cells as described previously. Cells were monitored daily for infection by observation at ten times magnification under UV light for infection. The results are summarised in table 3.2. In some cases the 293T cells fluoresced but co-cultured PM1 cells did not become infected. This suggests that the *nef*EGFP protein was expressed in the 293T cells however the *env* gene was deficient in some way and thus the resultant virus was not sufficiently fit to enter cells. From the table it can be seen that approximately half of the viruses did not infect PM1 cells. This suggests that a high proportion of the *env* genes investigated were defective in some way. This is consistent with previous reports which have found high levels of defective proviral genomes *in vivo* (Sanchez et al., 1997). It cannot be ruled out, however, that the high proportion of non-infectious viruses obtained was due to some flaw in this method of virus production. This will have to be fully investigated before firm conclusions about the proportion of defective *env*

	Fluorescence in 293T cells	Growth in PM1s
RO1	+	+
RO2	++	++
RO3	++	++
RO4	-	-
RO5	++	-
RO6	++	+
RO7	++	+
RO8	++	-
RO9	++	-
RO10	++	-
LT1	-	-
LT2	++	-
LT3	++	++
LT4	++	-
LT5	-	-
LT6	++	++
LT7	++	++
LT8	-	-
LT9	-	-
LT10	++	-

Table 3.2: Production of infectious virus from clones incorporating patient-derived *env* genes. ++: Fluorescence/infectious virus was present; +: Limited infectious virus with slow-growing phenotype; –: No fluorescence /infectious virus was observed.

genes are drawn.

3.3.3 Producing virus without amplifying DNA in E. coli

The method of chimeric virus production described above eliminates much of the selective pressure placed on viral genomes by standard cloning methods. This is due firstly to the use of limiting dilution PCR to generate *env* sequences. This prevents preferential primer binding, recombination during PCR and single nucleotide polymorphisms. In addition, retention of the first part of *env* from the lab adapted strain NL4.3 rather than cloning in patient-derived *env* genes should reduce the likelihood of cryptic promoter sequences being present. Nevertheless, a method which eliminated the need to use prokaryotic cells for amplification of the genome would be preferable. Such a method would allow production of viruses with *env* genes that more closely reflect the diversity present *in vivo*. Additionally, a method which did not require cloning in *E. coli* would reduce both the time taken to produce virus and the cost of the procedure. This could be particularly important for applications such as drug resistance assays (see discussion).

Three methods were devised to produce infectious virus using pNL4.3Δ*env*EGFP without DNA amplification in *E. coli*. The simplest of these involves carrying out a ligation reaction exactly as previously (figure 3.3), but using it directly to transfect 293T cells. This method will be referred to as non-cloning method 1. Ligation reactions were carried out using approximately 4 µg of digested *env* fragment and 4 µg of digested vector. As the *env* fragment is approximately 2.5 kb and the vector is approximately 7.5 kb this mixture represents a three fold molar excess of *env*. Two days after

transfection with the ligation mixture some 293T cells were seen to be fluorescent. The frequency of fluorescence was estimated to be 1 in 500 cells; ten-fold lower than that observed when 1 μg of *env* containing vector was used (data not shown). In addition, there was considerable evidence of cell toxicity; many cells died while others appeared more rounded than usual and became non-adherent. It was assumed that this was due to the large amount of DNA used for the transfection. The large quantity was necessary because during the ligation reaction many ligations would occur which would not produce DNA encoding the complete HIV genome. This non-HIV coding DNA would include cut vector fragments which had ligated to other cut vector fragments or *env* fragments which had ligated to other *env* fragments. These DNA fragments could not cause the cell to produce virus. It would be advantageous to devise a method in which a larger proportion of the DNA to be transfected would be capable of producing infectious virions. This would allow smaller quantities of DNA to be used and would thus lead to lower levels of toxicity. PM1 cells were co-cultured with the fluorescing 293T cells and evidence of infection was observed.

A variation on the ligation mixture method was designed to maximise the quantity of HIV-coding DNA used for transfection by amplifying fragments which had ligated in the desired manner (non-cloning method 2). The ligation mixture was used as template for two separate PCRs. The products of these were two overlapping fragments which together encoded the HIV coding sequence (figure 3.9). The larger fragment was amplified using a forward primer which annealed in the plasmid sequence upstream of the 5' HIV LTR

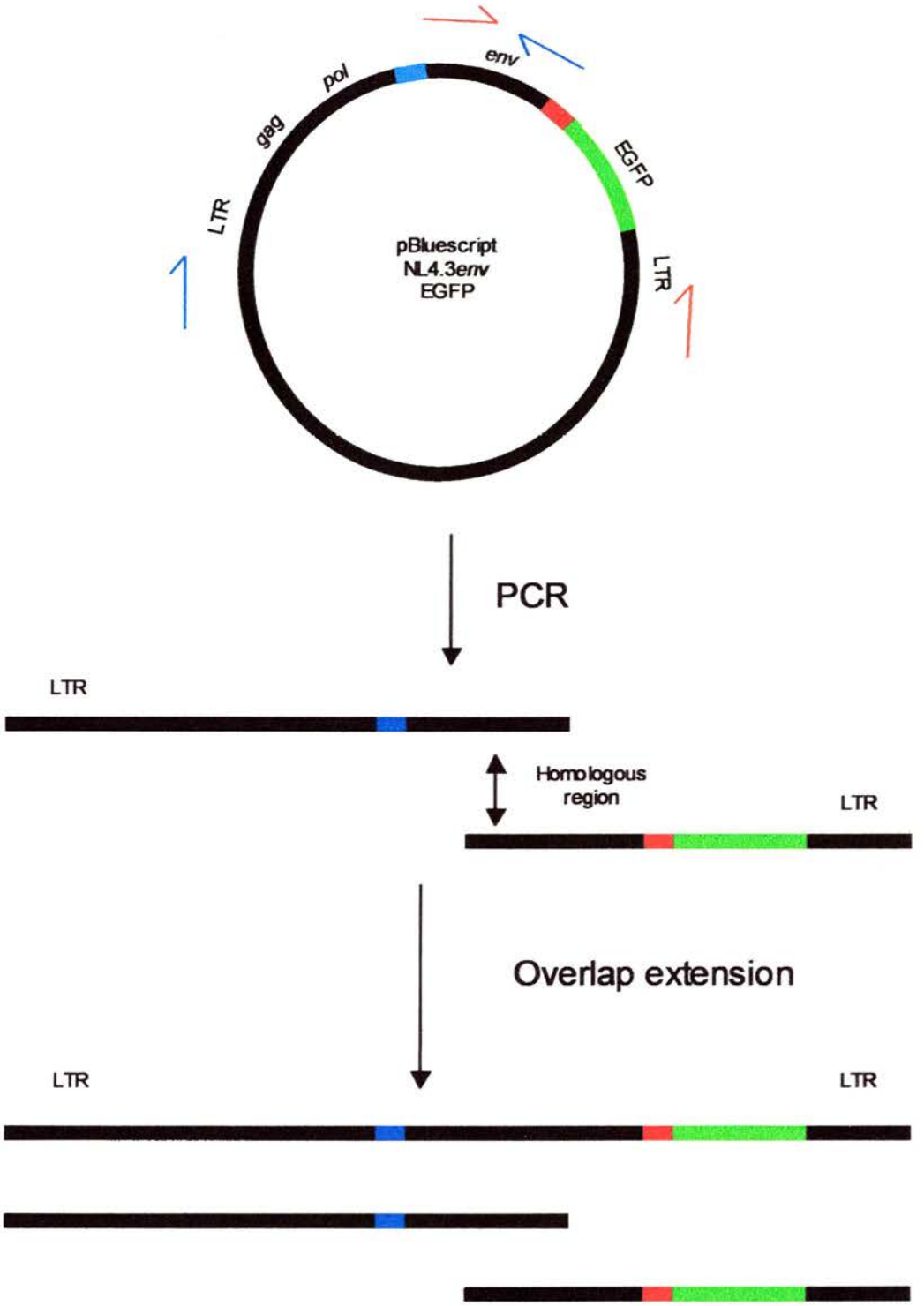


Figure 3.9: Overlapping fragments method of virus production (non-cloning method 2). Ligation was carried out as described previously and illustrated in figure 3.3. PCR was carried out using the ligation mix as template to produce two overlapping fragments which together encoded the entire HIV genome including LTRs. Note that fragments which had ligated at one end only could also serve as template. The two overlapping fragments were transfected into 293T cells.

and a reverse primer which annealed in *env* downstream of the V3 region. The smaller fragment was amplified using a forward primer which annealed in *env* upstream of the V3 region and a reverse primer which annealed in the plasmid sequence downstream of the 3' HIV LTR. Thus the two generated fragments had an overlap consisting of the entire V3 region. These fragments were subjected to an overlap extension reaction to generate full length viral genomes. This method is a variation on standard PCRs. The fragments were first mixed in PCR buffer and Taq and dNTPs added. The fragments were heated to separate the strands. The temperature was then lowered to allow annealing of single strands of DNA. In some cases, a strand from each of the different fragments will anneal in the overlapping portion. The temperature was then raised to an optimum temperature for Taq activity. In cases where strands from different fragments have annealed, second strand DNA synthesis will now proceed in a 5' to 3' direction producing a fragment encoding the entire HIV genome. The optimal number of required overlap extension cycles was investigated. Five cycles appeared to give the optimum amount of overlap extension product while additional cycles appeared to result in degradation of the DNA fragments (data not shown). The resultant DNA was used to transfect 293T cells. The product of the overlap extension reaction (approximately 8 µg), a mixture of fragments which had not been subjected to overlap extension (approximately 8 µg) or each of the fragments alone were used to transfect 293T cells. Either of the fragments in isolation did not result in fluorescence of the 293T cells. Surprisingly, after two days the overlap extension and the fragment mixture

wells contained similar numbers of fluorescent cells (approximately 1 in 500) (data not shown). This suggested that the major mechanism for the production of full length viral genomes was recombination within the transfected cells and not the overlap extension reaction. Levels of toxicity could not be accurately assessed for this experiment due to a fault in the incubator which led to a high level of cell death (see note in Appendix 1 for details). Co-culture of PM1 cells confirmed that infectious virus was present.

A less time consuming method of virus production utilising recombination within 293T cells was devised (figure 3.10) (non-cloning method 3). The use of a ligation reaction was eliminated. Instead PCR based techniques alone were used. The basis of the technique was to modify the PCR generated *env* fragment so that it would overlap at both ends with the cut vector. Both fragments could then be used to transfect 293T cells. The PCR generated *env* fragment already had a region of overlap with the cut vector at its 5' end; the portion which had removed by digestion with *Acc65* I in previous methods described. However the portion downstream of the *Xho* I restriction site was homologous with a region in *nef* which lies downstream of EGFP in pNL4.3Δ*env*EGFP. Thus, if recombination was allowed to take place at this end, the EGFP gene would be removed. It was decided to modify the *env* at its 3' end so that it ended with a region which overlapped with EGFP. First a fragment was produced which encompassed the start of *nef* and the entire EGFP gene as present in pNL4.3*env*EGFP. To generate this fragment, PCR was carried out using pNL4.3HXB2*env*EGFP as template and using a forward primer which annealed at the beginning of *nef* and a reverse primer

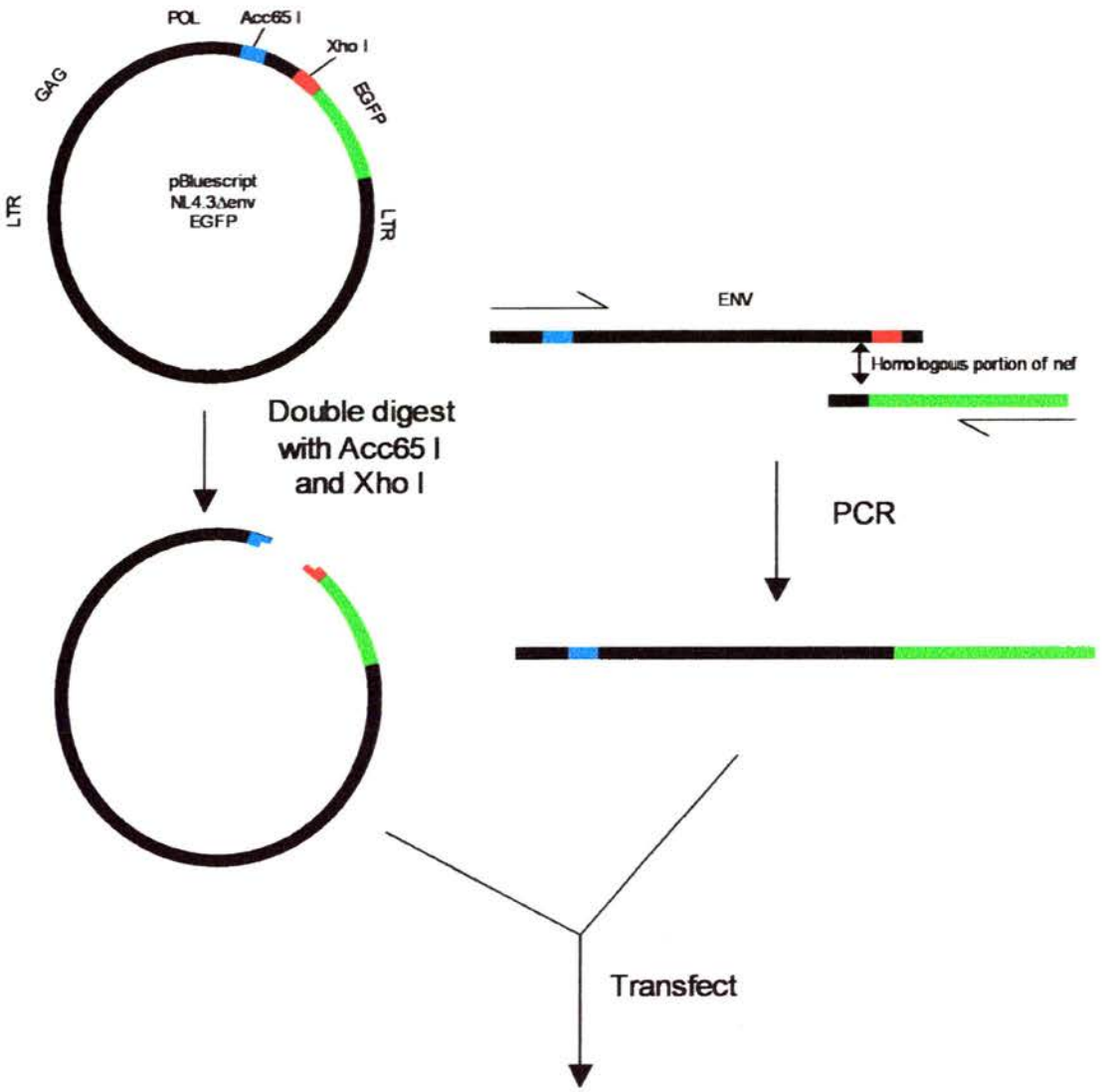


Figure 3.10: Second overlapping fragments method of virus production (non-cloning method 3). A DNA fragment was generated which encorporated the end of the *env* fragment (the first portion of the *nef* gene) and the EGFP fragment. A PCR was carried out using both this fragment and PCR generated *env* as template with primers positioned as shown. NL4.3Δ*env*EGFP was digested with *Acc65* I and *Xho* I. The cut vector and *env*EGFP fragment were transfected into 293 T cells.

which annealed at the end of EGFP. This fragment was purified and then mixed with an *env* fragment. PCR was carried out using this mixture as template. The forward primer annealed at the beginning of *env* while the reverse primer annealed at the end of EGFP. As the two templates had a region of overlap (the *nef* gene), after denaturation, some strands from the two different fragments would anneal. This would then provide a template for completion of the *env*EGFP fragment.

The purification method of the *nef*EGFP fragment was crucial in this process. When this fragment was spin column purified, the *env*EGFP fragment was produced by PCR even without the addition of an *env* fragment. This is because enough of the pNL4.3HXB2*env* plasmid remained to act as a template for this reaction. However if the *nef*EGFP fragment was gel purified then *env*EGFP was produced only when both the *env* and the *nef*EGFP fragments were present (data not shown). This suggests that in this case both fragments are being used as template. Transfection of the *envEGFP* fragment alone did not cause 293T cells to fluoresce (data not shown). 4 µg each of cut vector and *env*EGFP fragment were used to transfect 293T cells. After two day, similar levels of fluorescence to those seen in the other "non-cloning" methods were observed (data not shown). Again a high degree of toxicity was observed. Co-culture of PM1 cells confirmed that infectious virus was present.

The quantity of DNA it was possible to use without toxic effects was investigated (figure 3.11). The main toxic effect seemed to be due to the cut vector. Reducing the quantity from 1.2µg to 0.3 µg reduced the toxic effect

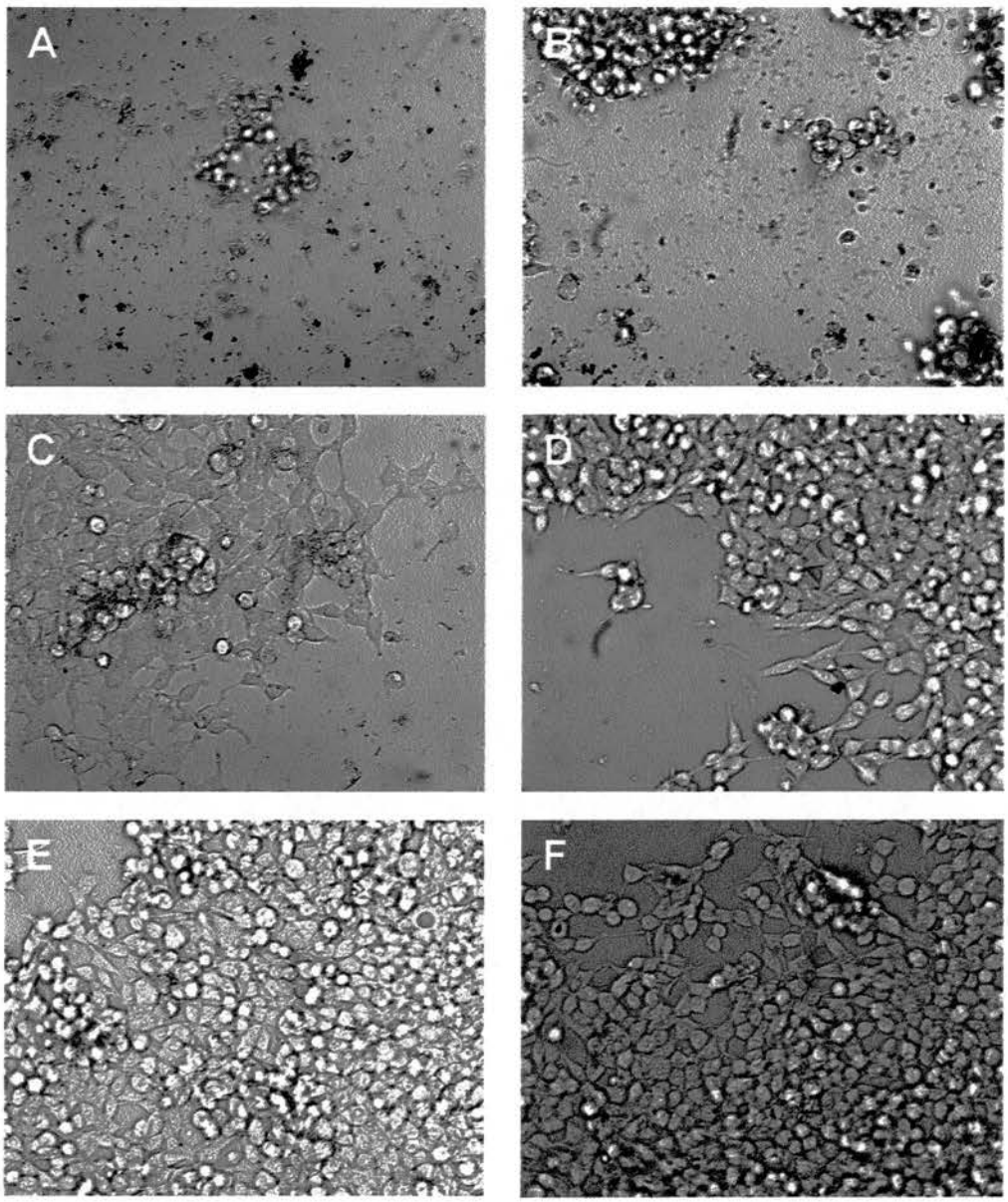


Figure 3.11: 293T cells one day after transfection viewed at 10X magnification under light microscope. Cell death is observed where a high concentration of cut vector- 1.2µg- is used (A and B). Reducing the quantity of cut vector to 0.3µg results in far less cell death (C). Media alone (D), Transfast reagent alone (E) or 1.3µg of envEGFP fragment (F) do not appear to cause significant cell death.

considerably. 1.2µg of the *env*EGFP fragment did not appear to cause significant toxic effect. Using these quantities of fragments reduced the amount of cell death observed. However this did result in a relatively low transfection efficiency as measured by the proportion of cells fluorescing after 2 days. Less than one cell in a 1000 appeared to be glowing. Next the quantity of *env*EGFP fragment required for optimum virus production was investigated. In addition to 0.3 µg of cut vector, either 0.9 or 1.5 µg of *env*EGFP fragment was used to transfect 293T cells and the proportion of fluorescing cells assessed after two days. Surprisingly, using the lower quantity of *env*EGFP resulted in a higher proportion of fluorescing cells. This could be due to a slight toxic effect exerted by the higher quantity.

An overview of the three methods of virus production is given in figure 3.12. Despite low efficiencies, all three methods of virus production described were capable of producing a variety of infectious viruses (table 3.3).

3.4 Discussion

In this chapter methods were investigated for the production of virus which could be used to characterise *env* phenotypes. A new vector, pNL4.3Δ*env*EGFP, was developed which enabled the production of chimeric viruses incorporating PCR amplified *env* genes. Recombinant DNA technology was used to insert *env* genes. Two factors in the method of virus production minimised artefactual selective pressure on the HIV genome. Firstly, the *env* genes were amplified at limiting dilution. This ensured that all the clones produced were essentially identical. If a mixed template is used for the *env* PCR then the clones will contain a library of different *env* sequences.

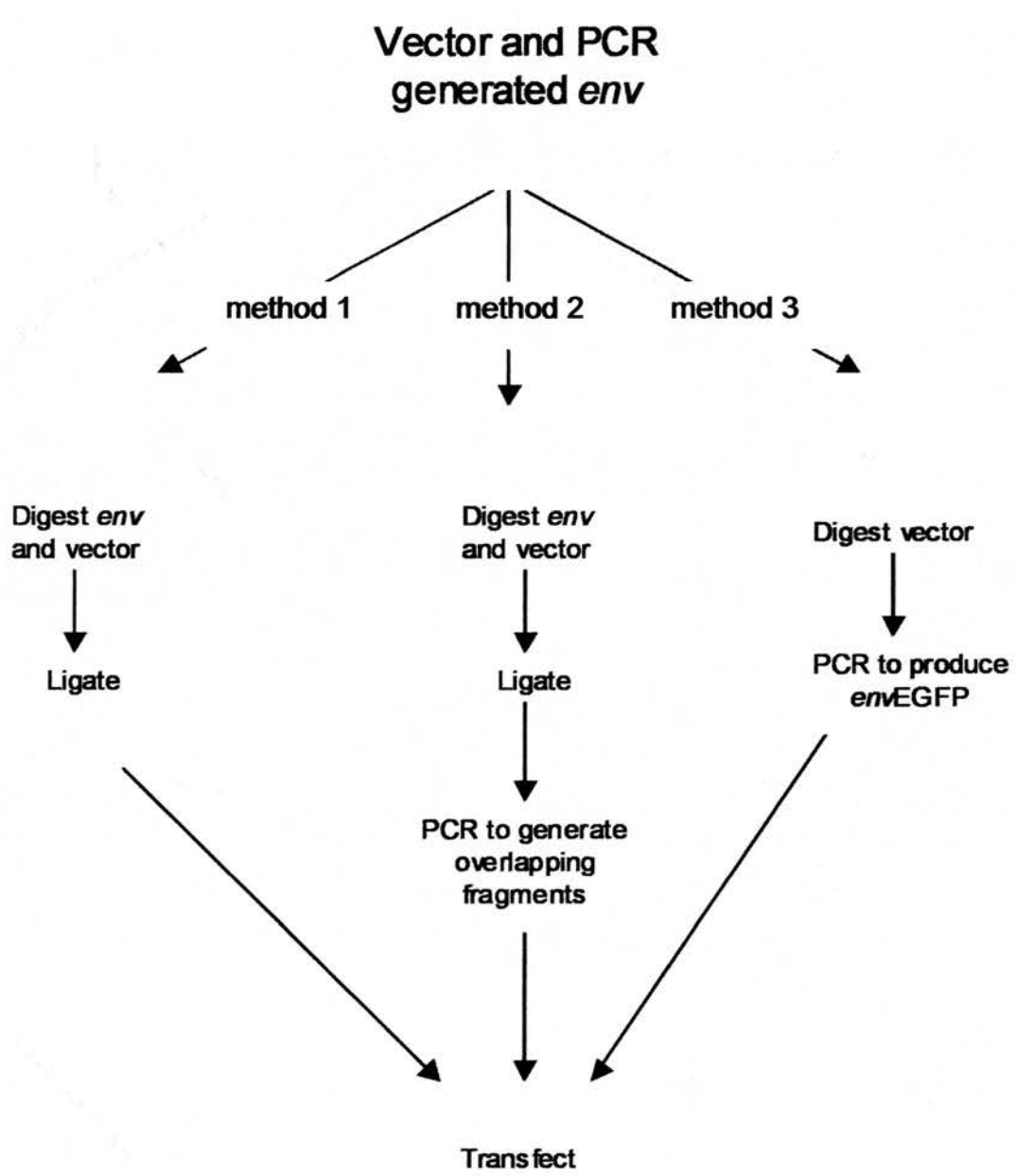


Figure 3.12: Overview of non-cloning methods used to produce infectious virus.

env	Method 1		Method 2		Method 3	
	293T	PM1	293T	PM1	293T	PM1
HXB2	+	+	+	+	+	+
ADA	+	+	+	+	+	+
Patient derived (RO1)	+	+	+	+	+	+
Subtype A	N/D		N/D		+	+
Subtype C					+	+

Table 3.3: Comparison of ability of different non-cloning methods to produce fluorescence in transfected 293T cells and co-cultured PM1 cells. Presence of fluorescence in PM1 cells indicates that infectious virus was present. Note that the methods requiring ligation can not be used to produce virus with non-b subtypes of HIV-1 as the required restriction sites are not present.

This could lead to fitter variants becoming over-represented. To obtain a number of clones which more accurately reflect the diversity of *env* present *in vivo*, a number of limiting dilution *env* products can be cloned separately. A second advantage this method has over some previously described methods is that the initial 139 bases of *env* are cleaved off before cloning. In viruses produced by this method, the fragment coding for the first part of *env* is derived from the NL4.3 backbone. As NL4.3 is a lab adapted strain, it is unlikely that this portion will contain cryptic promoters. Thus the chances of *env* being expressed in *E. coli* are minimised. This reduces the chance of growth in E. coli exerting any selective pressure on the viral genome.

This "cloning" method was used to produce infectious viruses incorporating *env* genes from four common lab adapted strains of HIV: NL4.3, HXB2, YU2 and ADA. Infection of cells by chimeric viruses could be monitored by FACS. When tested with co-receptor-expressing cell lines, these chimeric viruses had tropism profiles identical to the expected phenotype of the lab strains themselves. This confirms that the viruses have incorporated the exogenous *env* genes as expected. The cloning method was also used to produce virus incorporating *env* genes amplified from HIV-infected human DNA. Approximately half of these viruses were not replication competent in PM1 cells. This may reflect a high incidence of defective *env* genes *in vivo*. However, further work must be carried out to ensure that this result is not due to a defect in the method of virus production.

The cloning method described aims to minimise the selective pressure exerted on the viral genome by growth in *E. coli*. However, this selective

pressure is not eliminated. In particular, mutations which arise during PCR (i.e. due to Taq error) or during DNA replication in *E. coli* cells could confer a selective advantage to certain variants which could become overrepresented. A method which completely eliminated DNA amplification in E. coli would be preferable.

To this end, three new methods of virus production were attempted. The three methods are described fully previously. In brief the first method involved performing a ligation reaction and using this to transfect cells. The second method used the ligation reaction as template for a PCR which produced two overlapping fragments. These fragments were used to transfect cells. The third method involved modifying the *env* fragment so that it overlapped at both end with the cut vector. The modified *env* and cut vector were used to transfect cells.

All three methods were capable of producing infectious virus however none were particularly efficient. In the case of the first method, a large portion of the DNA used for transfection would not code for the HIV genome. In order to obtain enough DNA encoding the HIV genome for efficient virus production a large quantity of ligation reaction would have to be used to transfect the cells. Unfortunately, using a large quantity of DNA is toxic to the cells. The second and third methods rely on recombination within the cells to produce HIV encoding DNA. It appears that while this does occur, it is not sufficiently efficient to produce large quantities of virus. The virus produced by any of these methods could be amplified by growth in PM1 cells. However, this process will impose a selection pressure on the virus favouring any *de novo*

mutations which enhance fitness.

Further work is required to find a method which can produce sufficient virus but does not require amplification of DNA in *E. coli*. It is possible that slight modifications of one of the methods described could achieve this. For example, an overlap extension reaction could be carried out using the fragments obtained by the third method. These could be used as the template for a further PCR which amplified the entire HIV genome. A very high proportion of the resultant DNA fragments would encode the HIV genome. These could be used to transfect cells.

There are a number of potential research applications for a method which accurately assays the phenotype of *env* genes. For example, there has been much recent debate about whether different subtypes of HIV have different tropism profiles. Conflicting results have emerged regarding the relative abilities of different subtypes to infect Langherhan's cells. Two studies found that subtypes E and C were more able to infect these cells however two subsequent studies found no such differences (SotoRamirez et al., 1996; Essex et al., 1997; Pope et al., 1997; Dittmar et al., 1997). There have been some suggestions that the frequency of syncytium-inducing (SI) variants differs by subtype (Dewolf et al., 1994; Zhong et al., 1995). Subtype C in particular seems to have a low frequency of SI variants (Peeters et al., 1999). Some associations between co-receptor use and subtype have been reported. In one study CXCR4 viruses were underrepresented in subtype C viruses echoing the findings of less SI variants in this subtype. Additionally, dual-tropic viruses were absent from all subtype D viruses investigated

(Tscherning et al., 1998). One study has investigated the neurotropism of subtypes A and D (Zhang et al., 2001). Brain derived subtype A and D viruses were found to be more divergent than subtype B viruses when compared to spleen derived samples. A method able to produce viruses, contained *env* genes which reflected the diversity found *in vivo*, could be vital in clarifying these issues.

In addition to its value in tropism research, a method of chimeric virus production could have important clinical applications. Recently a number of potential therapeutic agents which inhibit HIV entry have been described. Currently, the most promising of these is a synthetic peptide known as T20. T20 binds to the gp41 portion of *env* and thereby inhibits its fusion with host membranes (Kilby et al., 1998). Phase three clinical trials of this agent are underway. Variants resistant to T20 have emerged in study subjects receiving T20 monotherapy (Wei et al., 2002). If T20 or other entry inhibitors becomes widely available as a treatment for HIV it will be important to monitor patients for the development of drug resistance. The chimeric virus technique described here could be used to achieve this. Chimeric viruses containing *env* amplified from patients could be grown in cells in the presence of the entry inhibitor. The presence of the EGFP gene in the viruses would provide a quick and easy way to identify resistant mutants.

Chapter 4: Results II

4.1 Introduction

In recent years the development and administration of clinically effective antiretroviral agents in the form of highly active antiretroviral therapy (HAART) have provided a valuable means of suppressing HIV replication in the peripheral blood and lymphoid systems of treated patients. These therapies have been successfully employed to extend the life of HIV infected individuals yet do not comprise a cure for infection because replication competent HIV persists in certain cellular and/or anatomical reservoirs from which it can re-emerge upon cessation of therapy (Davey, Jr. et al., 1999). One such potential viral reservoir or "sanctuary site" is within the central nervous system (CNS), where partially immunoprivileged conditions in conjunction with sub-optimal concentrations of antiretroviral agents could allow continued virus replication in spite of effective suppression in the periphery, circumstances that may also provide an ideal environment for the development of antiviral drug resistant viral variants. Considerable interest therefore exists in genetically characterising HIV-1 variants within the CNS of patients who have been treated with HAART

Therapeutic agents used to treat HIV infection must target the virus while having a limited effect on the host cell. Many processes in the HIV life cycle rely upon catalysis by essential host cell enzymes. Clearly inhibition of these enzymes cannot be used to reduce viral replication, as normal cell processes would also be affected. The HIV encoded enzymes are however suitable drug targets and indeed most currently licensed antiretrovirals inhibit either viral reverse transcriptase (RT) or protease (PR). A detailed description of

the function of these enzymes is given in section 1.5.2.

There are two main types of RT inhibitors; the nucleoside analogue RT inhibitors (NRTIs) and the non-nucleoside RT inhibitors (NNRTIs). The NRTIs include the drugs zidovudine (AZT), didanosine (ddI), stavudine (D4T) and lamivudine (3TC) (reviewed in Rang et al., 2003; Menendez-Arias, 2002). These exert their effect by acting as alternative substrates for RT. Once inside the cell they must be triphosphorylated by the host cell machinery before they are active. They can then compete with host cell deoxynucleotide triphosphate molecules (dNTPs) for incorporation into a growing DNA molecule. Once incorporated, the NRTIs act as chain terminators as they do not possess the hydroxyl group at the 3' end of their ribose moiety necessary for the attachment of another dNTP.

Resistance to this type of drug can occur due to mutations near the nucleotide binding site of RT that result in host dNTPs being bound preferentially to NRTIs. For example the M184V mutation results in a steric barrier to lamivudine binding (Sarafianos et al., 1999). This is because the β-branched amino acid valine is in opposition with the oxythioliane ring of the inhibitor. Mutations which confer resistance to NRTIs can also be found in areas of RT distal to the active site. Such mutations do not affect the ability of RT to bind NRTIs but rather increase the removal of the chain terminating nucleoside (Arion and Parniak, 1999; Meyer et al., 1999).

A group of agents closely related to the NRTIs, are the nucleotide RT inhibitors such as tenofovir. These compounds are already monophosphorylated and thus the conversion to a triphosphate form can

occur more quickly. Their mode of action is the same as the NRTIs and thus similar patterns of resistance mutations are likely.

The NNRTIs are a heterogeneous group of compounds which have a mode of action quite distinct to the NRTIs. They do not bind to the active site of the enzyme and thus are non-competitive inhibitors (reviewed in Rang et al, 2003; Menendez-Arias, 2002). Instead they bind to a hydrophobic pocket close to RT's active site and induce a conformational change in the protein structure which abrogates its catalytic activity. This class of therapeutic agents includes nevirapine, delavirdine, efavirenz and capravirine.

As the NNRTIs do not interact directly with the active site of the enzyme, mutations to their binding site may have a relatively small effect on viral fitness. Thus resistance to NNRTIs can appear quickly after the start of treatment. Indeed single amino acid changes can sometimes be sufficient to confer high-level resistance to certain NNRTIs (Balzarini, 1999). Some of the newer NNRTIs such as efavirenz and capravirine are less affected by such single amino acid changes. These agents attach to a number of conserved amino acids and thus multiple mutations at sites constrained by strong selective pressure are required to abrogate drug effectiveness (Ren et al., 2001; Ren et al., 2000).

In the past decade, a number of drugs have been developed which inhibit viral PR (reviewed in Rang et al, 2003; Menendez-Arias, 2002). These PR inhibitors (PIs) act on a late stage of the HIV life cycle; the cleavage of the viral proteins which allows viral maturation. Like the NRTIs, PIs are analogous to the normal substrate of PR and thus act as competitive

inhibitors. Examples of PIs are saquinavir, nelfinavir, indinavir, ritonavir and amprenavir.

Mutations which confer resistance to PIs, can occur either in the PR gene itself or sequences encoding the cleavage regions recognised by PR (reviewed in Menendez-Arias, 2002). Mutations to the active site of PR often reduce viral fitness significantly however further mutations elsewhere in PR can compensate for this. In addition, mutations at the cleavage sites in the GagPol polyprotein can result in an increased cleavage efficiency further compensating for mutations to the PR active site. Thus in order for the virus to become resistant to PIs and remain relatively fit, a number of mutations at different locations are required. This delays the emergence of variants with high-level resistance to these drugs (Molla et al., 1996; Zhang et al., 1997).

Drug resistance mutations can be classified according to their effect on phenotype (Hirsch, et al., 2000). Some mutations cause high level resistance to a given drug and are rapidly selected for. Such mutations have been termed primary or major mutations. Secondary, or minor, resistance mutations may contribute to resistance when primary mutations are present. Alternatively in some cases they counteract a loss of viral fitness caused by the acquisition of primary mutations.

Monotherapy with any other known anti-retroviral agent, is not effective for extended periods of time because resistant variants quickly emerge and predominate in treated individuals. This can be explained by the error prone replication of HIV in combination with the very high levels of viral turnover in infected individuals which allows extremely rapid viral evolution (see section

1.9.1). Concurrent treatment with a number of different anti-retroviral agents is far more effective at suppressing viral replication. The current treatment regime of choice is highly active retroviral treatment (HAART). This involves treatment with at least three drugs including either one or two NRTIs, a NNRTI and/or a PI (reviewed in Menendez-Arias, 2002; Weller, 1999).

As mentioned above, a major problem with HAART is that although viral replication is suppressed, the virus is never eliminated from certain cellular and anatomical reservoirs termed sanctuary sites. Thus once treatment is stopped, virus is still present to reseed infection of other sites. In addition a major concern is that if virus is able to replicate in areas where there are suboptimal levels of drugs, resistant variants could evolve.

One such sanctuary site is the CNS. Anti-retroviral agents are retarded from entry into the CNS to varying degrees with most appearing to have poor CNS penetration. Certain agents including zidovudine and indinovir have been reported to be able to enter the brain more efficiently but this may have been overestimated (reviewed in Groothuis and Levy, 1997). Some studies have measured the amount of drug in post mortem brain tissue and compared the levels with those found in plasma. Unfortunately, in most cases no account has been taken of the significant quantity of blood present in any piece of brain tissue. Thus much of the drug assumed to be present in the brain may in fact have been present in capillary blood vessels.

Other studies have measured the concentration of a given drug in the CSF. This however, will probably not represent the concentration of drug in the extra-cellular fluid (ECF) of most of the brain. Drugs moving from the CSF

into the ECF do so by diffusion and a gradient of concentration is created according to distance from the brain surface. The penetration of drugs from the CSF into the human brain ECF is thought to be between 1 and 1.5 cm from the brain surface (Blasberg et al., 1977). Thus the drug concentrations found in the CSF may only be found around cells very near to the brain surface.

Detailed calculations which take into account the transcapillary uptake and efflux of drugs reveal that the concentration of zidovudine likely to be found in the CNS of treated individuals is far lower than the concentration required to inhibit even the most zidovudine sensitive strains (Groothuis and Levy, 1997). This is in contrast to the oft-cited statistic that zidovudine levels in the CNS are approximately 60% of those found in the periphery. Studies of the uptake of PIs into the CNS are currently sparce. One study did calculate the uptake of indinavir across the blood brain barrier (Stuver et al., 1996). The authors reported a low uptake of the drug into the CNS although the uptake rates were higher than those reported for NRTIs.

In light of the poor CNS penetration of many drugs, a number of investigators have expressed a fear that the brain might provide a sanctuary site where drug-resistant variants can evolve. Various studies have addressed this by comparing the occurrence of drug resistance mutations in sequences from different sites.

Several studies have compared sequences of RT derived from the CSF and blood of zidovudine treated individuals. An early study found that concentrations of zidovudine in the CSF four hours after administration are

50% of those found in plasma (Klecker, Jr. et al., 1987). As mentioned previously, the concentration of zidovudine in the brain ECF is likely to be considerably lower. Thus the CSF does not necessarily represent the most likely sanctuary site for evolution of drug-resistance variants. Indeed most studies have found no differences in the distribution of mutations conferring resistance to zidovudine when sequences from the CSF and plasma are compared (Sei et al., 1996; Wildemann et al., 1993; Distefano et al., 1995).

Relatively little research has been done on the distribution of zidovudine resistance mutations in virus from the brain itself. One study obtained sequences of RT from proviral DNA extracted from brain tissue, spleen or lymph node (Wong et al., 1997). In three out of the four study subjects examined, a difference in distribution of zidovudine-resistance conferring mutations was observed between brain and spleen/lymph node. In these study subjects the sequences derived from the brain were more likely to be zidovudine sensitive compared to spleen/lymph-node sequences. A study which obtained RT sequences from RNA extracted from brain tissue, spleen and lymph node found the distribution of resistance mutations to be concordant within most individuals (McClernon et al., 2001). In four out of ten individuals who had taken zidovudine however, there was some degree of discordance in the distribution of resistance mutations. Again, in all these cases, the brain derived RT had fewer resistance-associated mutations.

Thus the extent to which the brain may act as a sanctuary site for viral replication during zidovudine treatment remains unclear. From the limited studies available it appears that in some cases the RT gene may contain

fewer resistance mutations in the brain than in the periphery. This could occur if levels of the drug were very low in the brain and thus exerted no selective pressure on the virus. It is possible that in some regions of the brain closer to the surface and therefore to the CSF, higher but still sub optimal levels of zidovudine may drive the evolution of resistant variants.

The distribution of mutations which confer resistance to PIs between the brain and the periphery is even less well characterised. Again, there is a possibility that sub-optimal concentrations of the drug may drive the evolution of drug resistant mutants. Alternatively, it is possible that if the drug is present at very low concentrations, there will be no drug pressure on the PR gene and therefore wild-type PR will be present in the brain.

Clearly further research is required to fully characterise the distribution of drug resistant mutations in HIV found in the brain.

4.2 Aims

The aim of this project was to characterise the distribution of drug resistance associated mutations in HIV proviral DNA isolated from post-mortem brain and lymphoid tissue.

4.3 Results

4.3.1 Selection of study subjects

The integrity of the blood brain barrier and thus the ability of virus and drugs to enter the CNS may differ depending on the stage of HIV-related illness. For this study two subjects who had died from AIDS-related illness and two who had died while still pre-symptomatic were chosen (tables 4.1 and 4.2).

Study subject	Age	Sex	Cause of death	Risk factor	No. of lymphocytes per µl blood		Time before death of last lymphocyte count
					CD4	CD8	
320	30	M	AIDS	IVDU	8	103	2m
369	31	M	AIDS	IVDU	23	440	7d
311	29	F	OD	IVDU	260	1432	18d
234	34	M	OD	IVDU	229	294	4m22d

Table 4.1: Clinical background of study subjects. OD: Over-dose.

Study subject	HIV related pathology				Other pathology	Proviral load per 10^6 cells	
	Atrophy	HIVE	p24GM	p24WM		Lymphoid	Brain
320	+	+	+	+	PML	2592	1150
369	-	+	-	-	CMV, lymphoma	102	40
311	-	-	-	-	none	81	887
234	-	-	-	-	none	9	3

Table 4.2: Brain pathology of study subjects. HIVE: HIV encephalitis; p24GM: p24 antigen detected in grey matter by immunohistochemistry; p24WM: p24 antigen detected in white matter by immunohistochemistry; PML: Progressive multifocal leukoencephalopathy; CMV: Cytomegalovirus.

The first criterion for selection of subjects was that they must have taken anti-retroviral drugs for a minimum of one year (figure 4.1). Additionally, subjects considered to have a relatively high chance of harbouring distinct viral variants in the brain and lymphoid system were chosen. Thus this was not a random sample of subjects but rather a case study of subjects who were thought most likely to harbour distinct patterns of drug resistance between compartments. The subjects who had died with AIDS-related illness were selected on the grounds that pathological evidence of HIVE had been found. This condition is associated with compartmentalisation of virus between brain and the lymphoid system (Wang et al., 2001). A previous study (Marsden et al, in press) had identified two pre-symptomatic study subjects (234 and 311) in whom V3 sequences derived from the brain were phylogenetically distinct from those present in the lymphoid system. Thus these subjects were also chosen for inclusion.

4.3.2 Method of limiting dilution PCR

PCR amplification using a genetically heterogeneous template may result in products which do not accurately reflect the *in vivo* situation. Certain variants may be preferentially amplified due to higher affinity with the primers used for PCR potentially leading to an over-representation of minor variants. Partially synthesised product can dissociate from its template during the denaturation step and then re-anneal to a different template molecule. This can lead to artefactual recombinant products (Meyerhans et al., 1990). In order to obtain unadulterated PCR products amplified from a single template molecule, PCRs were carried out at limiting dilution (Simmonds et al., 1990) (section

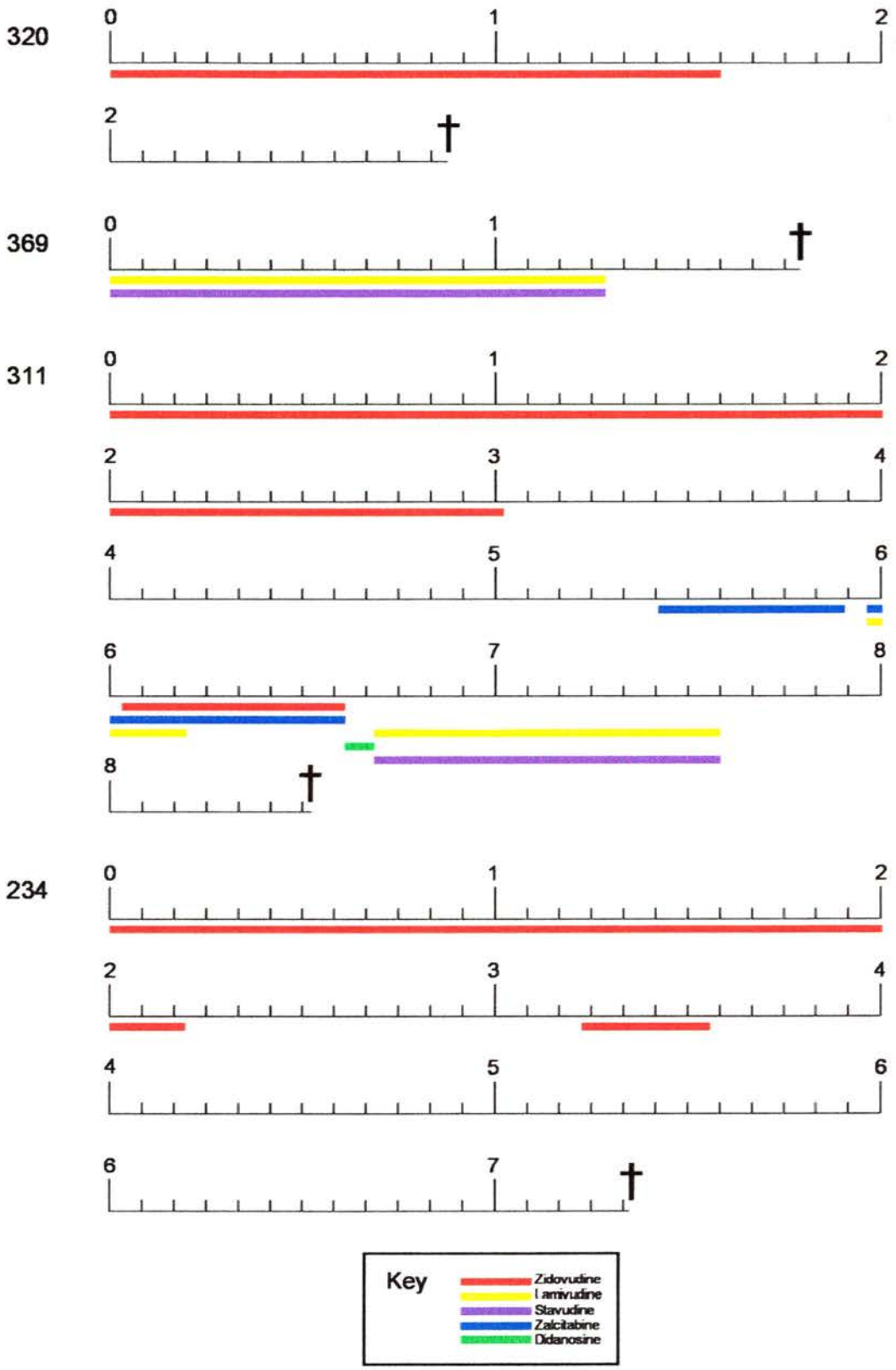


Figure 4.1: Timelines showing antiretroviral drug regimes of study subjects. Numbers indicate years since initiation of treatment. Coloured bars below timelines indicate periods of time when antiretroviral drug was taken. † Indicates death of patient.

2.1.5). In this technique the template DNA is diluted to a point at which more than half of the PCRs carried out will be negative. It is assumed that any reactions which are positive have proceeded using a single molecule as template. In practice, a large number of PCR reactions must be carried out for every limiting dilution product obtained (table 4.3 and figure 4.2).

4.3.3 V3 sequencing

The most variable region of the HIV genome is the V3 region of gp120 and this makes it useful in studies of viral evolution. Comparison of V3 sequences from brain and lymphoid tissue can indicate if a separately replicating viral population is present in the brain. Note that segregation of V3 sequences between brain and the lymphoid system does not guarantee that *pol* sequences will be similarly distinct as discordant phylogenies between different proviral regions are common (Morris et al., 1999). However, the presence of distinct sequences indicates a degree of genetic compartmentalisation between HIV variants in the brain and lymphoid system has occurred and thus it is more likely that distinct patterns of drug resistance mutations will be found. If the V3 sequences group phylogenetically with lymphoid derived sequences, it is likely that they have been amplified from virus present in contaminating blood in the brain sample or that they represent recent viral infiltration.

Between five and eight limiting dilution V3 PCR products were obtained from brain and lymphoid tissue of study subjects 150 and 207. The V3 primers used produced a 324 bp fragment (figure 4.3A). These products were sequenced and phylogenetic trees produced (figure 4.4). The predicted

Study subject	Tissue	Fragment	Total number of PCRs	Number of limiting dilution products
320	Lymph node	V3	45	5
	Brain	V3	45	8
	Lymph node	P-B	100	8
	Brain	P-B	40	8
369	Lymph node	V3	45	8
	Brain	V3	88	5
	Lymph node	P-B	40	8
	Brain	P-B	190	7
	Lymph node	PR	60	8
	Brain	PR	204	8
311	Lymph node	P-B	63	8
	Brain	P-B	83	8
	Lymph node	PR	80	8
	Brain	PR	80	8
234	Lymph node	P-B	45	7
	Brain	P-B	63	8
		Total	1271	120

Table 4.3: Numbers of PCR reactions carried out and products obtained for each tissue sample.

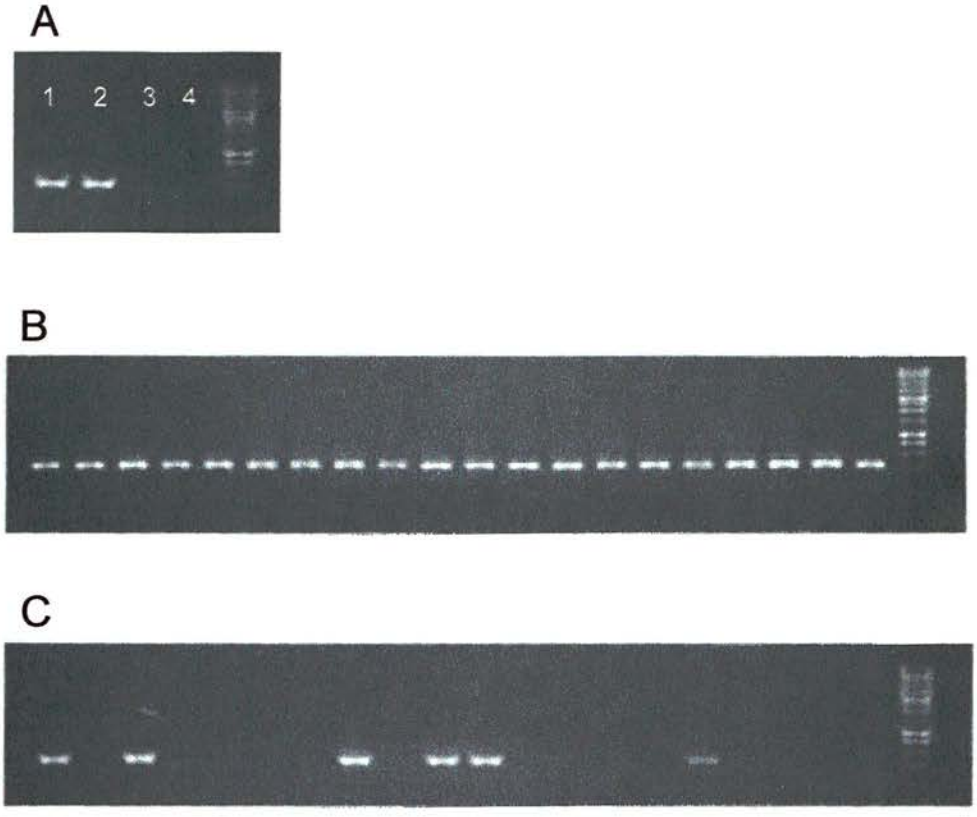


Figure 4.2: Obtaining limiting dilution PCR products- representative gel images. A: Nested PCRs were carried out on serial ten fold dilutions of the extracted DNA (lane 1: undiluted; lane 2: 10^{-1}; lane 3: 10^{-2}; lane 4: 10^{-3}). B: Twenty replicate nested PCRs were carried out using template at concentration estimated to yield less than 8 positive reactions out of twenty. Typically, a dilution of 1/3 of the last positive ten fold dilution was used initially. Thus in this case, template DNA at a concentration of 1/3 x 10^{-1} was used. All PCR reactions carried out using template at this concentration were positive and so further replicates were attempted at a lower concentration. C: Twenty replicate nested PCRs were carried out using template at concentration of 10^{-2}. Six reactions were positive and these products were thus assumed to be amplified from a single template molecule. Further replicate reactions were carried out using DNA at the same concentration.

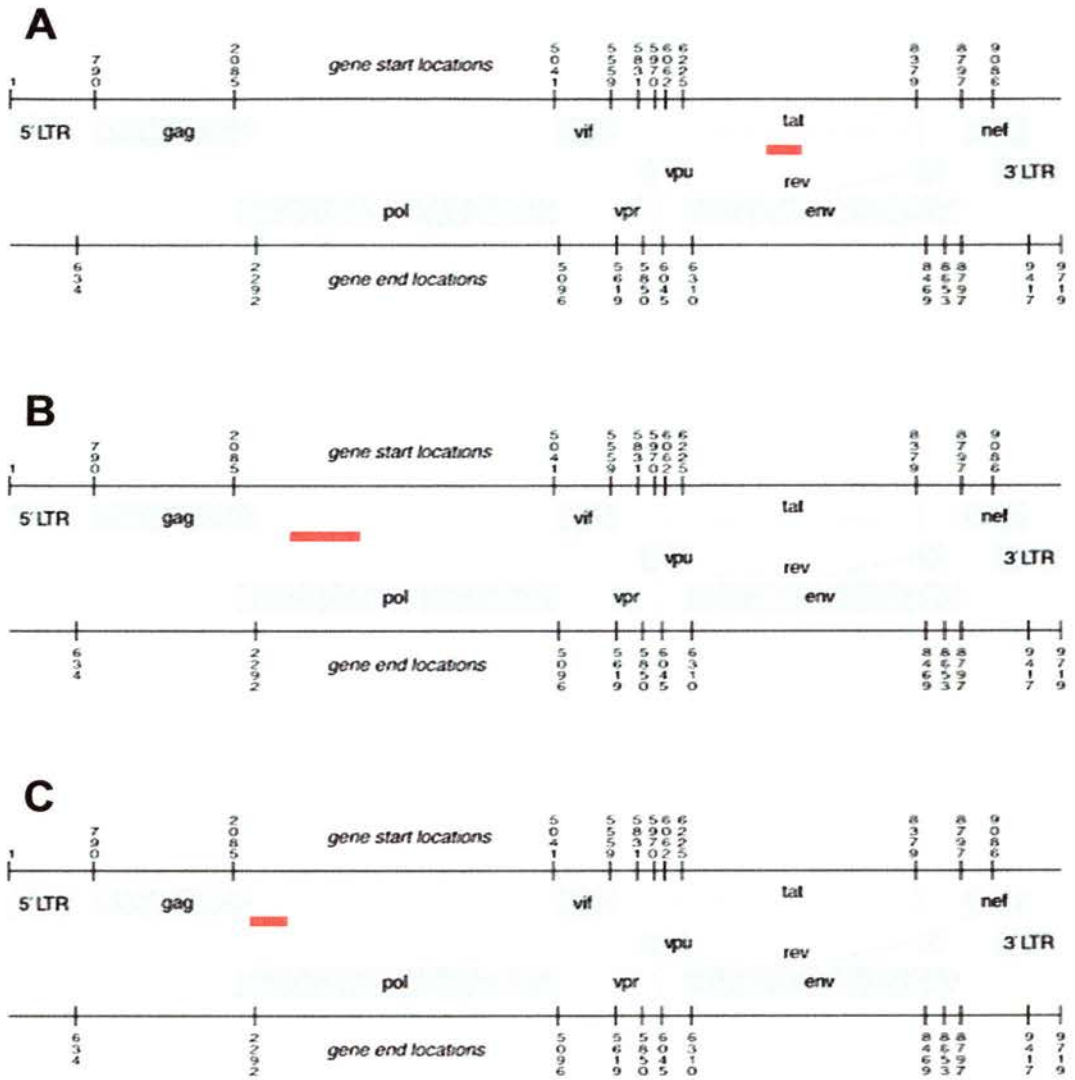


Figure 4.3: Location of PCR fragments (shown here in red) within HIV genome. A: V3 fragment (nucleotides 7009 to 7333) B: RT fragment (nucleotides 2622 to 3252) Note that two overlapping fragments amplified from a single primary product were used to obtain the full sequence. C: PR fragment (nucleotides 2253 to 2578) All nucleotide positions are relative to the HXB2 genome.

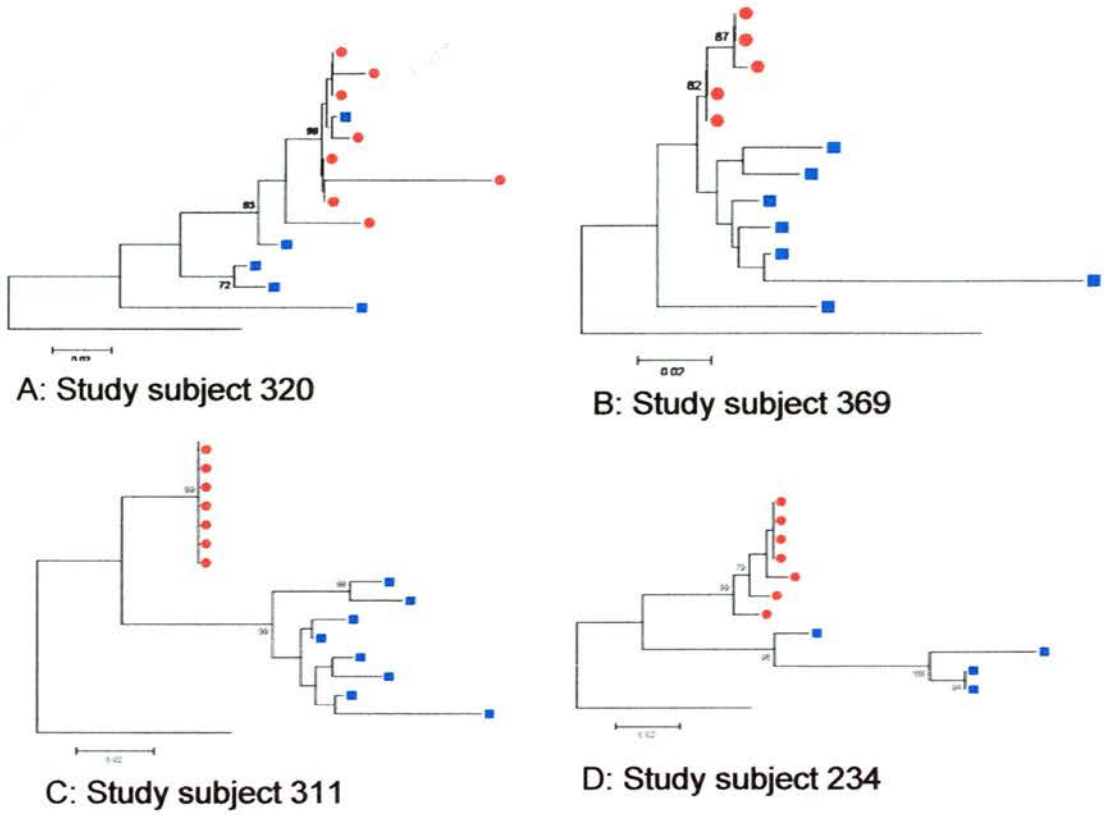


Figure 4.4: Phylogenetic trees constructed using V3 sequences amplified at limiting dilution. Distances between sequences were calculated using the Jukes-Cantor distance correction method (distances indicated on scales below trees). The distance matrix was used to construct trees using the neighbour-joining method. The robustness of observed groupings was tested by bootstrap resampling and values over 70 are indicated on the trees. The V3 sequence of the subtype B lab variant HXB2 was used as an outgroup to root the trees. Red circles represent brain-derived sequences. Blue squares represent lymphoid tissue-derived sequences. Trees for subjects 311 and 234 were obtained from Matt Marsden (Marsden et al., in press).

amino acid sequences of the sequenced fragments are shown (figure 4.5). V3 sequencing had been carried out previously by Matt Marsden for study subject 234 and 311 and the amino acid alignments and phylogenetic trees obtained are also included here for reference (Marsden et al., in press). Only one sequence in the entire data set (311L-1, Fig 4.5) included a basic amino acid at either position 11 or 25 within the V3 loop, a characteristic that is indicative of X4 variants. Therefore consistent with a large number of previous reports all brain derived sequences within this study had a predicted CCR5 using, macrophage tropic viral phenotype.

In three of the four study subjects the V3 tree topologies provided strong evidence for genetic compartmentalisation between brain and lymphoid derived sequences. Study subjects 369, 311, and 234 each had brain derived sequences that clustered together in monophyletic, bootstrap supported lineages, which were distinct from all lymphoid sequences obtained from the same individuals. Brain derived V3 sequences from subject 320 also clustered together into a single lineage separate from the majority of lymphoid sequences. However in this case the presence of a lymph node derived sequence within the brain lineage reduced the level of sequence segregation according to tissue as compared with the other study subjects.

A clear divergence between lymphoid and brain derived V3 sequences formed part of the criteria for the inclusion of study subjects 234 and 311.

4.3.4 RT sequencing

The region of RT used for sequencing was 630 bps and was encoded by two overlapping PCR fragments amplified from a single primary PCR product

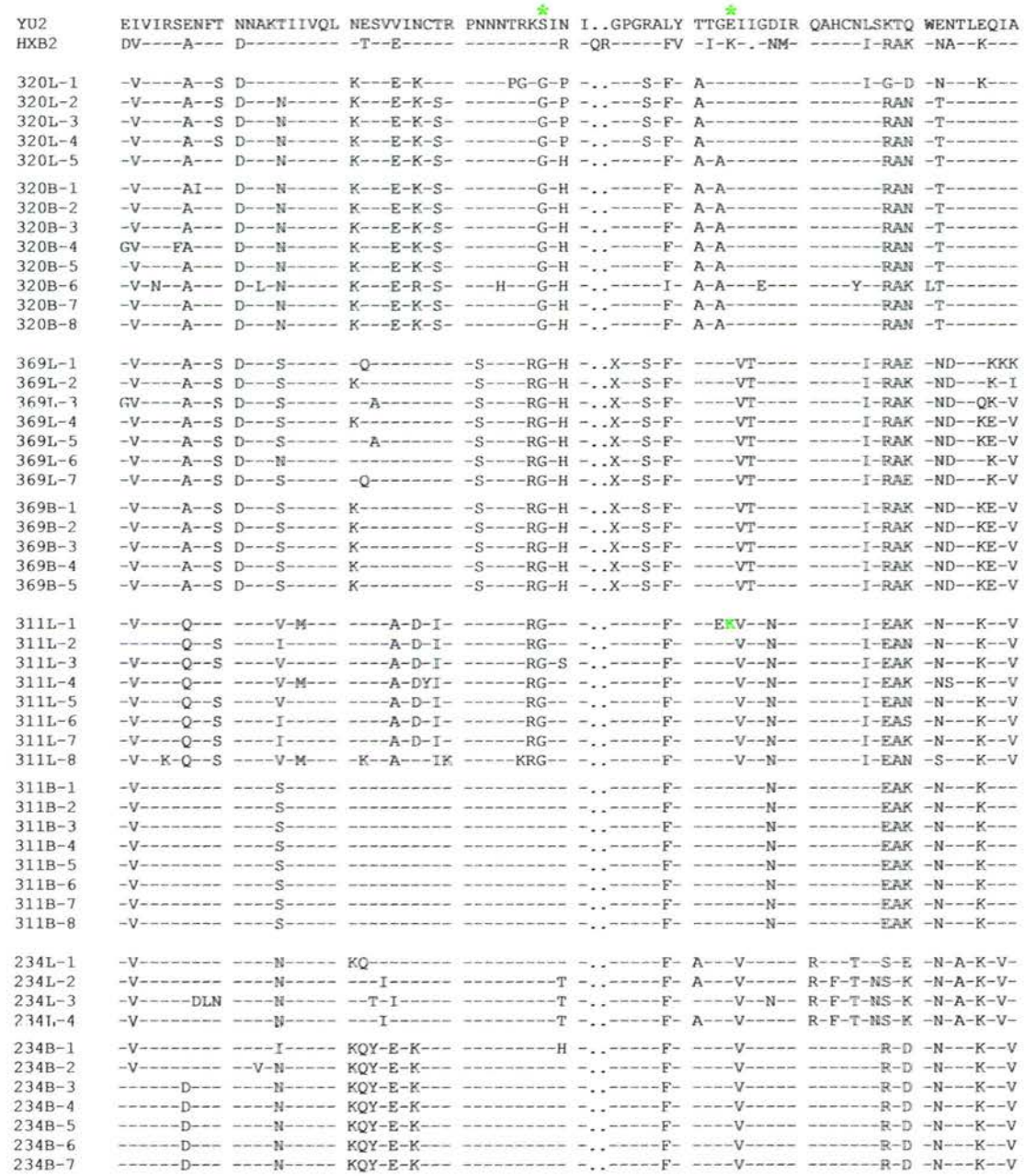


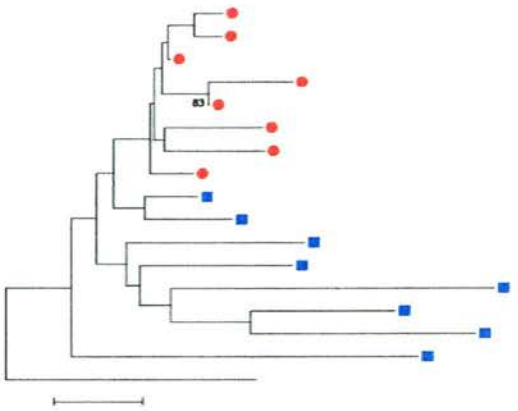
Figure 4.5: Predicted V3 amino acid sequences of PCR products amplified at limiting dilution from post-mortem tissue. L: Lymphoid tissue derived; B: Brain tissue derived. Green asterisks indicate sites at which the occurrence of positively charged amino acids is strongly correlated with CXCR4 usage. Dashes indicate identical bases. Dots indicate an alignment gap.

(figure 4.2B). Between seven and eight limiting dilution copies of each of the two fragments were obtained from brain and lymphoid tissue for each subject. The products were sequenced, aligned and phylogenetic trees produced (figure 4.6).

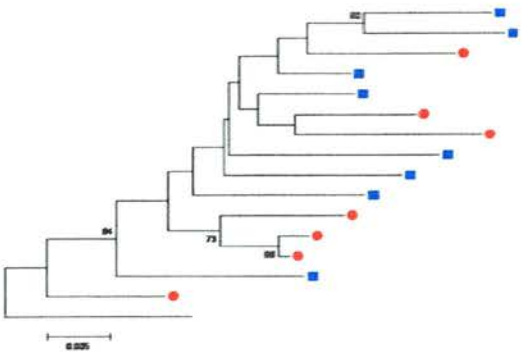
The tree topology for subject 320 suggests a high degree of genetic compartmentalisation between sequences amplified from brain or lymphoid tissues. All brain-derived sequences cluster together in a monophyletic lineage. Lymphoid-derived sequences form three lineages, all distinct from the brain sequences. In contrast, the tree for subject 369 provides little evidence of genetic compartmentalisation; brain and lymphoid derived sequences appear to be completely intersperced. The tree topologies for subjects 311 and 234 indicate an intermediate degree of genetic compartmentalisation. In both cases the brain-derived sequences form a polyphyletic group composed of three distinct lineages (two are boot-strap supported for subject 311 while all three are boot-strap supported for subject 234). No lymphoid-derived sequences are found in any of these lineages however lineages containing lymphoid-derived sequences are intersperced with "brain lineages".

The Los Alamos HIV drug resistance database was used to identify putative resistance-conferring mutations in the amino acid sequences (figure 4.7). This information is summarised in table 4.4.

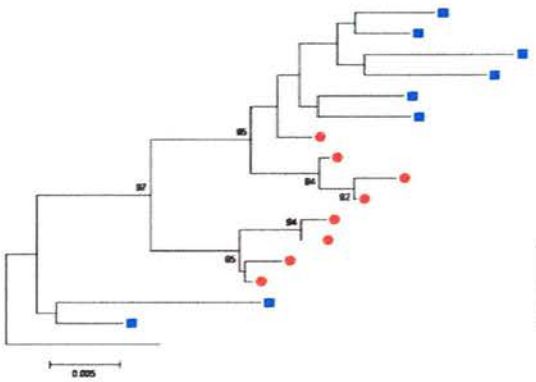
There were no examples of mutations conferring resistance to an NRTI which had not been prescribed to the subject from which the sequence was amplified. For example, no mutations conferring a high degree of resistance



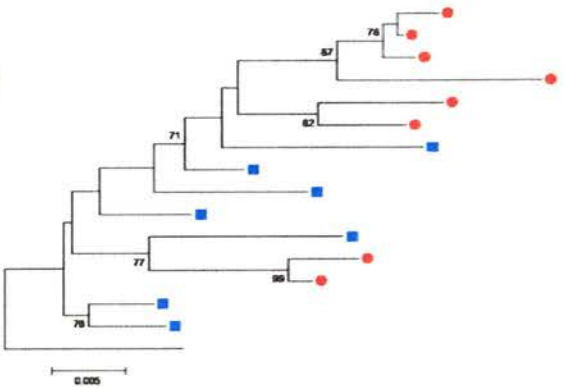
A: Study subject 320



B: Study subject 369



C: Study subject 311



D: Study subject 234

Figure 4.6: Phylogenetic trees constructed using RT sequences amplified at limiting dilution. Trees were produced as outlined in figure 4.4. Red circles represent brain-derived sequences. Blue squares represent lymphoid tissue-derived sequences.

Figure 4.7: Predicted RT amino acid sequences of PCR products amplified at limiting dilution from post-mortem tissue. L: Lymphoid tissue derived; B: Brain tissue derived. Dashes indicate identical bases. Dots indicate an alignment gap. Stars indicate a stop codon. Mutations conferring low to intermediate zidovudine and stavudine resistance and minor lamivudine resistance, indicated by **red** arrows, are **M41L** (Larder and Kemp, 1989); **L210W** (Gurusinghe et al., 1995) and **T215Y** (Larder and Kemp, 1989). (Bazmi et al., 2000; Taylor et al., 2000; Garcia-Lerma et al., 2003; Zhang et al., 1994) Mutation conferring minor zidovudine, stavudine and lamivudine resistance, indicated by an **orange** arrow is: **V118I** (Hertogs et al., 2000). Mutations which confer resistance to lamivudine but cause hypersensitivity to zidovudine, indicated by **green** arrows are: **K65R** (Tisdale et al., 1997) and **M184V** (Tisdale et al, 1997). Mutation data obtained from the Stanford HIV Drug resistance Database (Rhee et al. 2003). Note that figure continues over four pages.

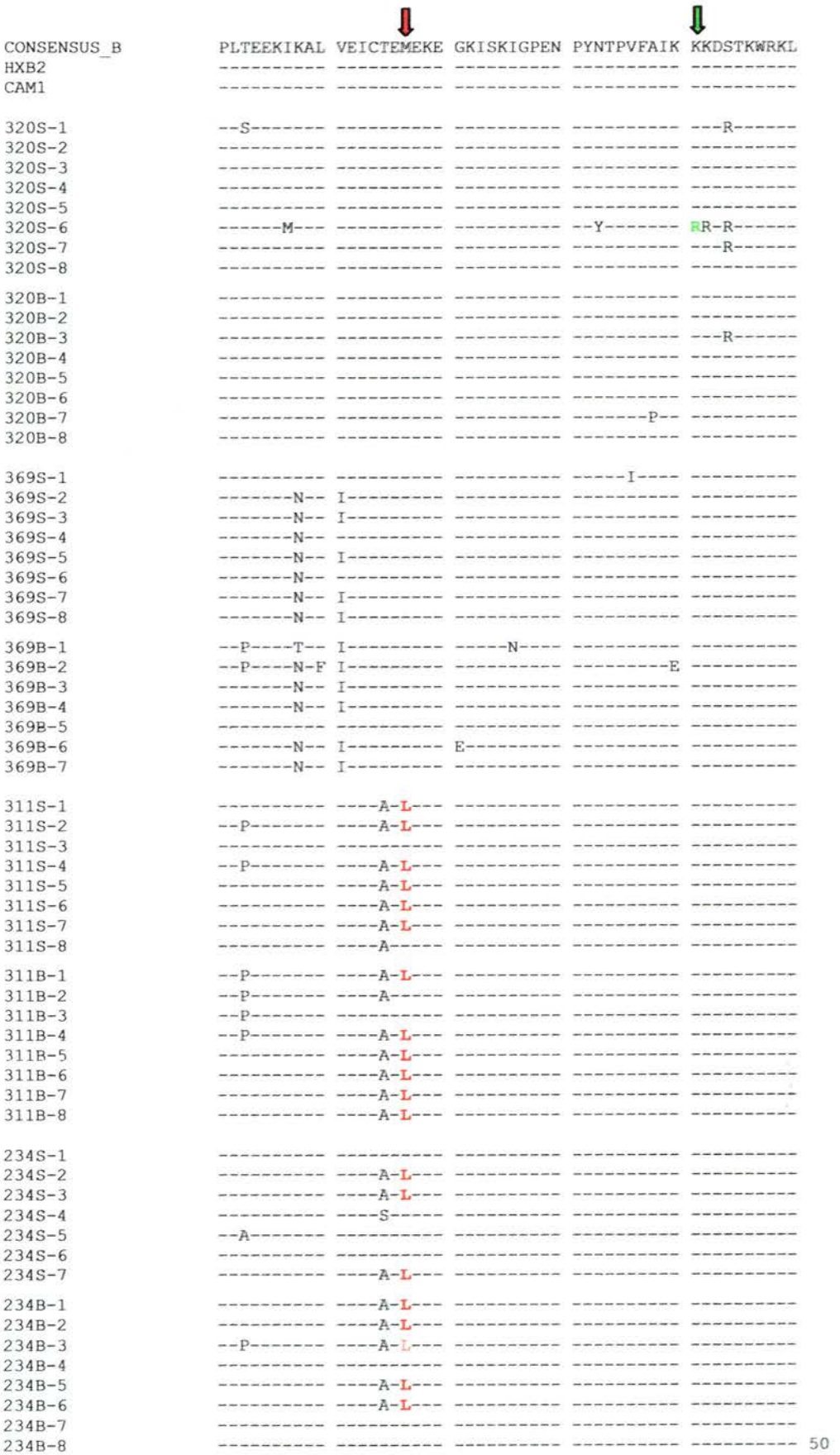

CONSENSUS_B PLTEEKIKAL VEICTEMEKE GKISKIGPEN PYNTPVFAIK KKDSTKWRKL
HXB2 ---------- ---------- ---------- ---------- ----------
CAM1 ---------- ---------- ---------- ---------- ----------
320S-1 --S------- ---------- ---------- ---------- ---R------
320S-2 ---------- ---------- ---------- ---------- ----------
320S-3 ---------- ---------- ---------- ---------- ----------
320S-4 ---------- ---------- ---------- ---------- ----------
320S-5 ---------- ---------- ---------- ---------- ----------
320S-6 ------M--- ---------- ---------- --Y------- RR-R------
320S-7 ---------- ---------- ---------- ---------- ---R------
320S-8 ---------- ---------- ---------- ---------- ----------
320B-1 ---------- ---------- ---------- ---------- ----------
320B-2 ---------- ---------- ---------- ---------- ----------
320B-3 ---------- ---------- ---------- ---------- ---R------
320B-4 ---------- ---------- ---------- ---------- ----------
320B-5 ---------- ---------- ---------- ---------- ----------
320B-6 ---------- ---------- ---------- ---------- ----------
320B-7 ---------- ---------- ---------- -------P-- ----------
320B-8 ---------- ---------- ---------- ---------- ----------
369S-1 ---------- ---------- ---------- -----I---- ----------
369S-2 -------N-- I--------- ---------- ---------- ----------
369S-3 -------N-- I--------- ---------- ---------- ----------
369S-4 -------N-- ---------- ---------- ---------- ----------
369S-5 -------N-- I--------- ---------- ---------- ----------
369S-6 -------N-- ---------- ---------- ---------- ----------
369S-7 -------N-- I--------- ---------- ---------- ----------
369S-8 -------N-- I--------- ---------- ---------- ----------
369B-1 --P----T-- I--------- -----N---- ---------- ----------
369B-2 --P----N-F I--------- ---------- ---------E ----------
369B-3 -------N-- I--------- ---------- ---------- ----------
369B-4 -------N-- I--------- ---------- ---------- ----------
369B-5 ---------- ---------- ---------- ---------- ----------
369B-6 -------N-- I--------- E--------- ---------- ----------
369B-7 -------N-- I--------- ---------- ---------- ----------
311S-1 ---------- ----A-L--- ---------- ---------- ----------
311S-2 --P------- ----A-L--- ---------- ---------- ----------
311S-3 ---------- ---------- ---------- ---------- ----------
311S-4 --P------- ----A-L--- ---------- ---------- ----------
311S-5 ---------- ----A-L--- ---------- ---------- ----------
311S-6 ---------- ----A-L--- ---------- ---------- ----------
311S-7 ---------- ----A-L--- ---------- ---------- ----------
311S-8 ---------- ----A----- ---------- ---------- ----------
311B-1 --P------- ----A-L--- ---------- ---------- ----------
311B-2 --P------- ----A----- ---------- ---------- ----------
311B-3 --P------- ---------- ---------- ---------- ----------
311B-4 --P------- ----A-L--- ---------- ---------- ----------
311B-5 ---------- ----A-L--- ---------- ---------- ----------
311B-6 ---------- ----A-L--- ---------- ---------- ----------
311B-7 ---------- ----A-L--- ---------- ---------- ----------
311B-8 ---------- ----A-L--- ---------- ---------- ----------
234S-1 ---------- ---------- ---------- ---------- ----------
234S-2 ---------- ----A-L--- ---------- ---------- ----------
234S-3 ---------- ----A-L--- ---------- ---------- ----------
234S-4 ---------- ----S----- ---------- ---------- ----------
234S-5 --A------- ---------- ---------- ---------- ----------
234S-6 ---------- ---------- ---------- ---------- ----------
234S-7 ---------- ----A-L--- ---------- ---------- ----------
234B-1 ---------- ----A-L--- ---------- ---------- ----------
234B-2 ---------- ----A-L--- ---------- ---------- ----------
234B-3 --P------- ----A-L--- ---------- ---------- ----------
234B-4 ---------- ---------- ---------- ---------- ----------
234B-5 ---------- ----A-L--- ---------- ---------- ----------
234B-6 ---------- ----A-L--- ---------- ---------- ----------
234B-7 ---------- ---------- ---------- ---------- ----------
234B-8 ---------- ---------- ---------- ---------- ---------- 50

Figure 4.7.

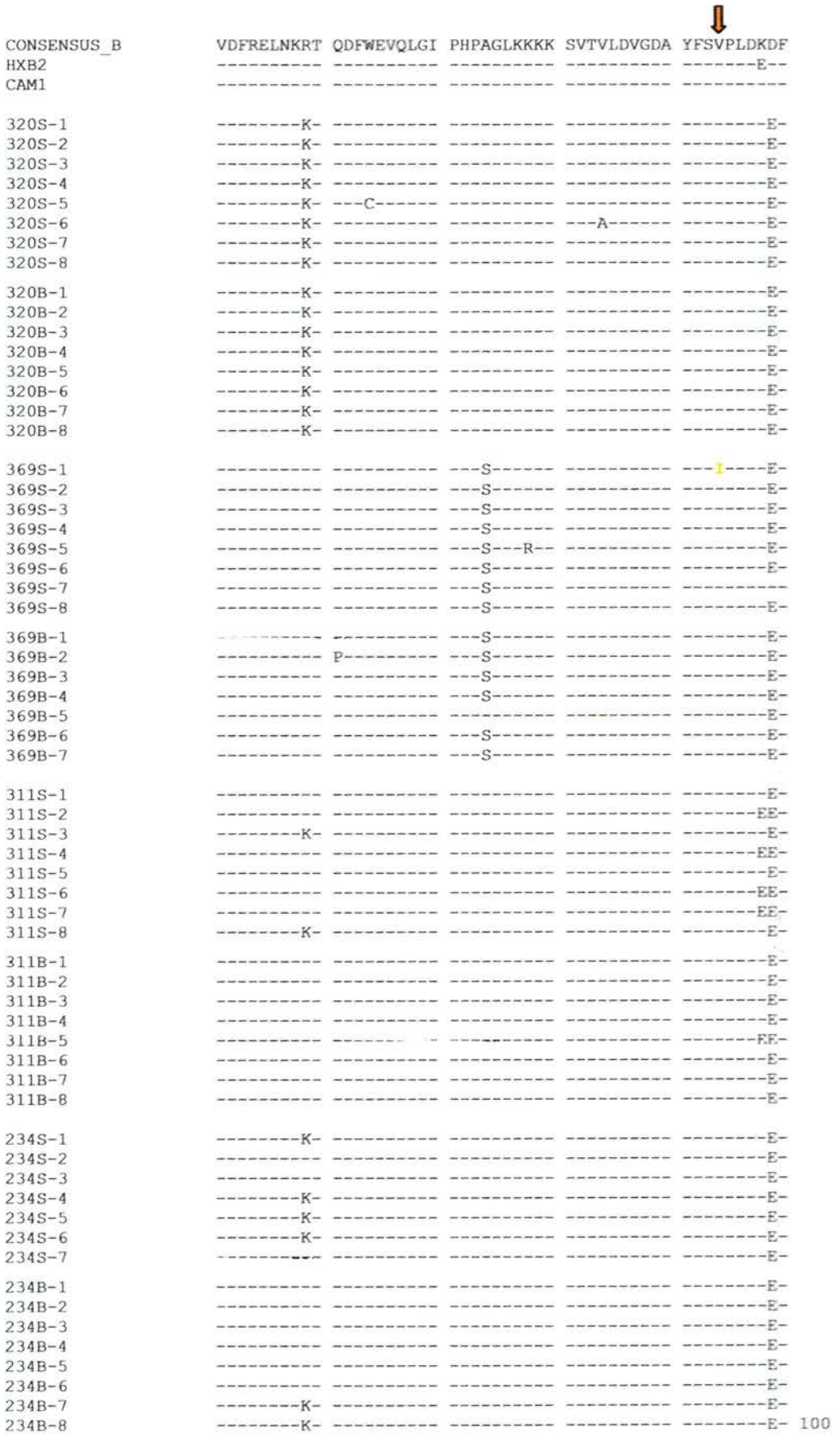


Figure 4.7: Continued

```
CONSENSUS_B         RKYTAFTIPS INNETPGIRY QYNVLPQGWK GSPAIFQSSM TKILEPFRKQ
HXB2                ---------- ---------- ---------- ---------- ----------
CAM1                ---------- T--------- ---------- ---------- ----------

320S-1              ---------- ---------- -I-------- -------C-- ----------
320S-2              ---------- ---G------ -I-------- -------C-- --------G-
320S-3              ---------- ---G------ ---------- -------Y-- ----------
320S-4              ---------- V--G------ ---------- -------Y-- ----------
320S-5              ---------- ---------- ---------- -------Y-- ----------
320S-6              ---A------ ---------- ---------- -------Y-- ----------
320S-7              ---------- V--------- ---------- -------C-- --------R-
320S-8              ---A------ ---------- ---------- -------C-- ----------

320B-1              ---------- ---------- ---------- -------Y-- ----------
320B-2              ---------- ---G------ ---------- -------Y-- ----------
320B-3              ---A------ ---G------ ---------- -------Y-- ----------
320B-4              ---------- ---------- ---------- -------Y-- ----------
320B-5              --*------- ---------- ---------- -------Y-- ----------
320B-6              G-*------- ---------- ---------- -------Y-- ----------
320B-7              ---A---T-- ---------- ---------- -------Y-- ----------
320B-8              ---A---T-- ---------- ---------- -------Y-- ----------

369S-1              ---------- R--------- ---------- ---------- ----------
369S-2              ---------- R--------- ---------- ---------- ----------
369S-3              ---------- R--------- ---------- ---------- ----------
369S-4              ------S--- R--G------ ---------- ---------- ----------
369S-5              ---------- R--------- ---------- ---------- ----------
369S-6              ---A------ R--------- ---------- ---------- ----------
369S-7              ---A------ R--------- ---------- E--------- ----------
369S-8              ---A---T-- R--------- ---------- ---------- ----------

369B-1              ---------- R--G------ ---------- ---------- ----------
369B-2              ---------- R--------- ---------- ---------- ----------
369B-3              ------S--- R--------- ---------- ---------- ----------
369B-4              ---A------ R--G------ ---------- -------G-- ----------
369B-5              ---------- R--------- ---------- ---------- ----------
369B-6              ---------- R--------- ---------- ---------- ----------
369B-7              ---------- R--------- ---------- ---------- ----------

311S-1              ---------- ----A----- ---------- ---S---C-- ----D-----
311S-2              ---------- ---------- ---------- ---S---C-- ----D-----
311S-3              ---------- ---------- ---------- -------A-- -R--D-----
311S-4              ---------- ---------- ---------- ---S---C-- ----------
311S-5              ---------- ---------- ---------- ---S---C-- ----------
311S-6              ---------- ---------- ---------- -------C-- ----------
311S-7              ---------- ---------- ---------- ---S------ -I--------
311S-8              ---------- ---------- ---------- ---------- I---------

311B-1              ---------- ----A----- ---------- ---S---C-- ----------
311B-2              ---------- ---------- ---------- ----V----- ----------
311B-3              ---------- ---------- ---------- ----V----- ----------
311B-4              ---------- ----A----- ---------- ---S---C-- ----------
311B-5              ---------- ---------- ---------- ----V----- ----------
311B-6              ---------- ---------- ---------- ---S---C-- ----------
311B-7              ---------- ---------- ---------- ---S---C-- ----------
311B-8              ---------- ---------- ---------- ----V----- ----------

234S-1              ---------- ---------- ---------- -------C-- I---------
234S-2              ---------- ---------- ---------- ----V----- ----------
234S-3              ------P--- ---------- ---------- ---------- -R--------
234S-4              ---------- ---------- ---------- ---------- I---------
234S-5              ---------- ---------- ---------- -------D-- -R--------
234S-6              ---------- ---------- ---------- ---------- ----D-----
234S-7              ---------- -------F-- ---------- ---------- -I--------

234B-1              ---------- ----A----- ---------- ---S---C-- ----------
234B-2              -R----P--G ----A----- ---------- ---S------ ----------
234B-3              ---------C ---------- ---------- ----V----- ----------
234B-4              ---------- ---------- ---------- ----V----- ----------
234B-5              ---------- ----A----- ---------- ---S---C-- ----------
234B-6              ---------- ----A----- ---------- ---S---C-- ----------
234B-7              ---------- ---------- ---------- -------D-- -R--------
234B-8              ---------- ---------- ---------- -------D-- -R-------- 150
```

Figure 4.7: Continued

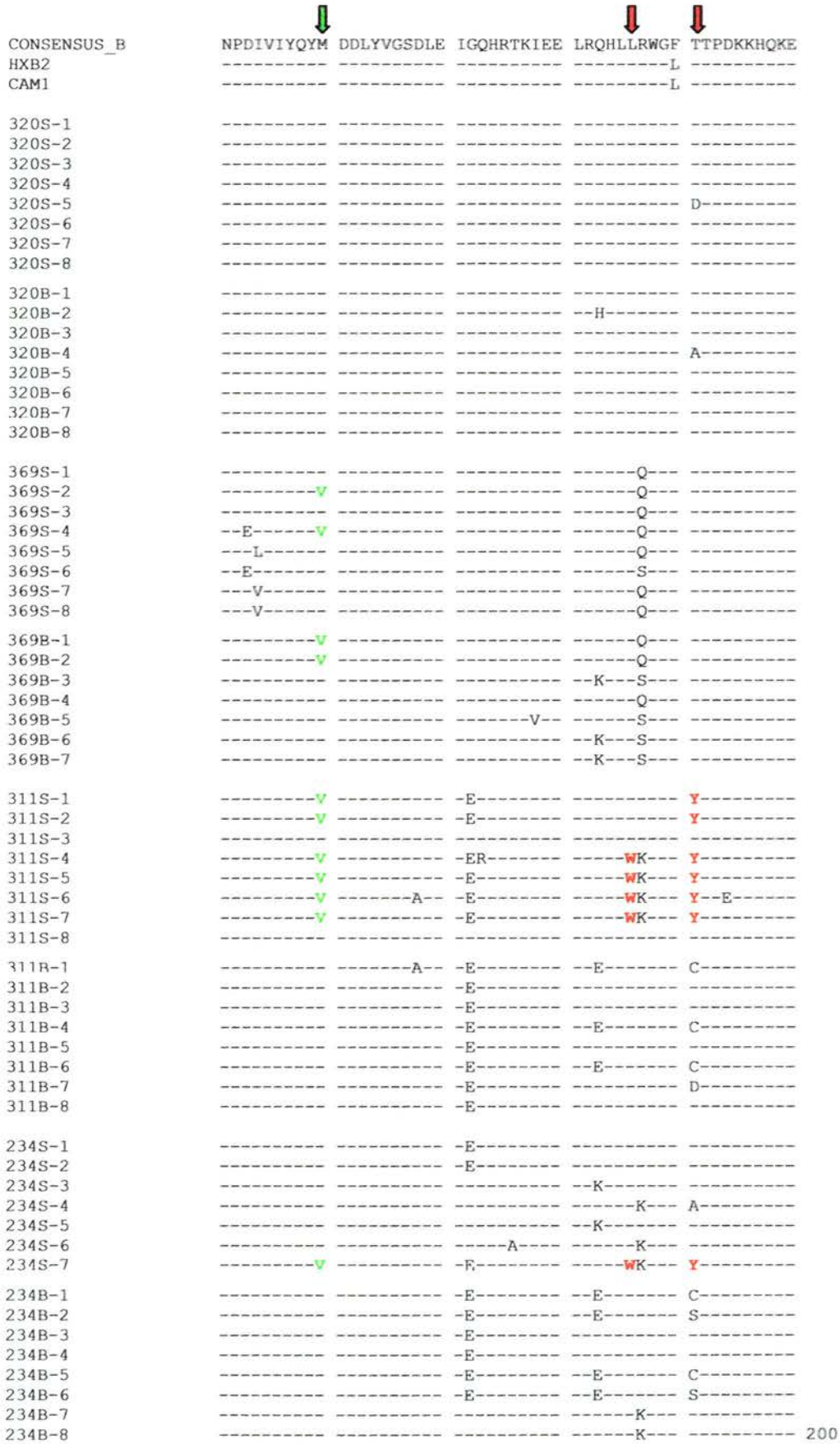


Figure 4.7: Continued

Subject	Tissue	No	M41 →L	V118 →I	L210 →W	T215 →Y	K65 →R	M184 →V	AZT PFR	Mean	3TC PFR	Mean
320	L	1							1 (169)	1	1 (142)	2.3
		2							1 (169)		1 (142)	
		3							1 (169)		1 (142)	
		4							1 (169)		1 (142)	
		5							1 (169)		1 (142)	
		6					●		0.6 (15)		11.1 (14)	
		7							1 (169)		1 (142)	
		8							1 (169)		1 (142)	
	B	1							1 (169)	1	1 (142)	1
		2							1 (169)		1 (142)	
		3							1 (169)		1 (142)	
		4							1 (169)		1 (142)	
		5							1 (169)		1 (142)	
		6							1 (169)		1 (142)	
		7							1 (169)		1 (142)	
		8							1 (169)		1 (142)	
369	L	1		●					1.5 (6)	1	1 (213)	40.1
		2						●	0.7 (141)		138 (142)	
		3							1 (169)		1 (142)	
		4						●	0.7 (141)		138 (142)	
		5							1 (169)		1 (142)	
		6							1 (169)		1 (142)	
		7							1 (169)		1 (142)	
	B	1						●	0.7 (141)	0.9	138 (142)	35.3
		2						●	0.7 (141)		138 (142)	
		3							1 (169)		1 (142)	
		4							1 (169)		1 (142)	
		5							1 (169)		1 (142)	
		6							1 (169)		1 (142)	
		7							1 (169)		1 (142)	
		8							1 (169)		1 (142)	
311	L	1	●			●		●	5.7 (42)	9.3	200 (41)	142.3
		2	●			●		●	5.7 (42)		200 (41)	
		3							1 (169)		1 (142)	
		4						●	0.7 (141)		138 (142)	
		5	●		●	●		●	20 (31)		199.5 (30)	
		6	●		●	●		●	20 (31)		199.5 (30)	
		7	●		●	●		●	20 (31)		199.5 (30)	
		8							1 (169)		1 (142)	
	B	1	●						4 (4)	3.3	6 (1)	4.8
		2							1 (169)		1 (142)	
		3							1 (169)		1 (142)	
		4	●						4 (4)		6 (1)	
		5	●						4 (4)		6 (1)	
		6	●						4 (4)		6 (1)	
		7	●						4 (4)		6 (1)	
		8	●						4 (4)		6 (1)	
234	L	1							1 (169)	4.6	1 (142)	30.8
		2	●						4 (4)		6 (1)	
		3	●						4 (4)		6 (1)	
		4							1 (169)		1 (142)	
		5							1 (100)		1 (147)	
		6							1 (169)		1 (142)	
		7	●		●	●		●	20 (31)		199.5 (30)	
	B	1	●						4 (4)	2.9	6 (1)	4.1
		2	●						4 (4)		6 (1)	
		3	●						4 (4)		6 (1)	
		4							1 (169)		1 (142)	
		5	●						4 (4)		6 (1)	
		6	●						4 (4)		6 (1)	
		7							1 (169)		1 (142)	
		8							1 (169)		1 (142)	

Table 4.4: Summary of resistance mutations present in RT sequences. L: lymphoid; B: Brain; AZT: zidovudine; 3TC: lamivudine; PFR: Predicted Fold Resistance- values are the median susceptibility of all isolates from the Stanford HIV drug resistance database containing the mutations shown and no other major drug-resistance mutations. Number of isolates tested to obtain median susceptibility values shown in brackets. Drug resistance mutations are colour coded as follows: **Red**: Low to intermediate AZT and D4T resistance; minor 3TC resistance. **Orange**: Minor AZT, D4T and 3TC resistance. **Green**: Confers hypersensitivity to AZT and D4T and intermediate resistance to 3TC. Mutations data obtained from the Stanford HIV Drug resistance Database (Rhee et al., 2003).

to zidovudine were found in subject 369 who had not taken this drug. Thus, the most likely explanation for the resistance mutations seen in the sequences is that evolution of the virus took place within the host in response to selective pressure from RT inhibitors. However, it cannot be ruled out that some of the mutations could have been acquired through transmission of a drug resistant variant; Indeed a study carried out in America revealed that between 1996 and 2000, approximately 10% of newly acquired HIV infections were with a virus possessing at least one NRTI-resistance conferring mutation (Little et al., 2002).

There was no difference in the pattern of drug resistance-conferring mutations between sequences derived from the brain and lymphoid tissue of subject 320 despite the sequences forming separate lineages according to tissue origin on the phylogenetic tree. Examination of the nucleotide sequence revealed that the observed phylogenetic split between the groups of sequences is largely due to segregating polymorphisms at two key nucleotide positions; at both position 363 and 393 the lymphoid-derived sequences have a guanine residue while the brain-derived sequences have an adenine residue (data not shown). Interestingly, both nucleotide changes are synonymous. Abrogation of these two discordant nucleotides results in phylogenetically interspersed sequences. All the sequences amplified from subject 320 possessed the R83K mutation. This mutation has been linked to NRTI resistance in one study (Masquelier et al., 1999) however another study found the mutation to be negatively related to RT inhibitor usage (CeccheriniSilberstein et al., 4 A.D.). Thus the *in vivo* significance of this

mutation is unclear. It is possible that the mutation is capable of conferring either sensitivity or resistance depending on the other mutations present in the sequence.

The brain and lymphoid tissue derived sequences from subject 369 also show no differences in the distribution of resistance mutations. Only one drug-resistance mutation is represented at all (M184V) and this appears in two lymphoid tissue and two brain derived sequences. As mentioned previously, this mutation confers resistance to lamivudine. The finding of no difference in drug-resistance conferring mutations between brain and lymphoid tissue is compatible with the interspersed nature of these sequences on the phylogenetic tree.

The only subject for which there was a substantial difference in drug-resistance mutation patterns between compartments was subject 311. One mutation conferring resistance to zidovudine (M41L) was fairly evenly distributed in sequences from both compartments. However two mutations were over-represented in the lymphoid derived sequences. These mutations were L210W and T215Y which confer resistance to zidovudine. Interestingly, two other mutations which may be associated with RT inhibitor use were also over-represented in the lymphoid-derived sequences. These are K122E which is associated with RT inhibitor use but does not appear to contribute significantly to resistance and R11K which confers resistance to zidovudine + atevirdine (data not included in table 4.4).

At first glance, the drug-resistance/ mutation pattern of subject 234 appears to differ between compartments however, this is largely due to the presence

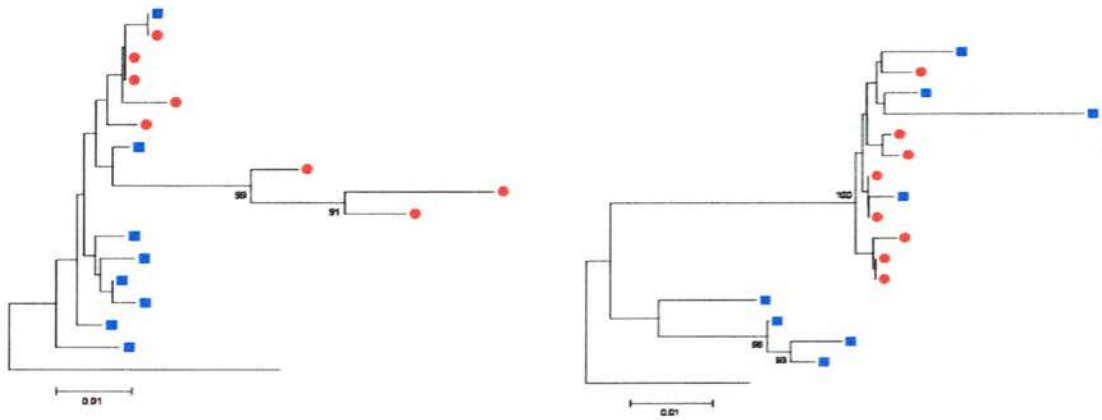
of one lymphoid tissue-derived variant (234L-7) which possesses multiple drug resistance mutations. If this sequence is removed then the distribution of drug resistance and drug associated mutations is fairly equal between compartments. Further sequences would need to be obtained to determine if sequence L7 represents a significant population of highly drug-resistant variants in the lymphoid compartment.

PR sequencing

Of the four study subjects two (369 and 311) were believed to have taken PIs. Thus the distribution of resistance mutations in the PR gene was examined in sequences amplified from these subjects. The region of PR used for sequencing was 325 bps long (figure 4.2C). Eight limiting dilution copies of the fragment were obtained from brain and lymphoid tissue for each subject. The products were sequenced, aligned and phylogenetic trees produced (figure 4.8).

The tree topologies for both subjects suggest a degree of genetic compartmentalisation. In both cases, all brain-derived sequences cluster in single monophyletic lineages (boot-strap supported in the case of subject 311). However, the presence of lymphoid-derived sequences within these lineages reduces the degree of genetic segregation according to tissue of origin.

The Los Alamos HIV drug resistance database was used to identify putative resistance-conferring mutations in the amino acid sequences (figure 4.9). This information is summarised in table 4.5.



A: Subject 369

B: Subject 311

Figure 4.8: Phylogenetic trees constructed using PR sequences amplified at limiting dilution. Trees were produced as outlined in figure 4.4. Red circles represent brain-derived sequences. Blue squares represent lymphoid tissue-derived sequences.

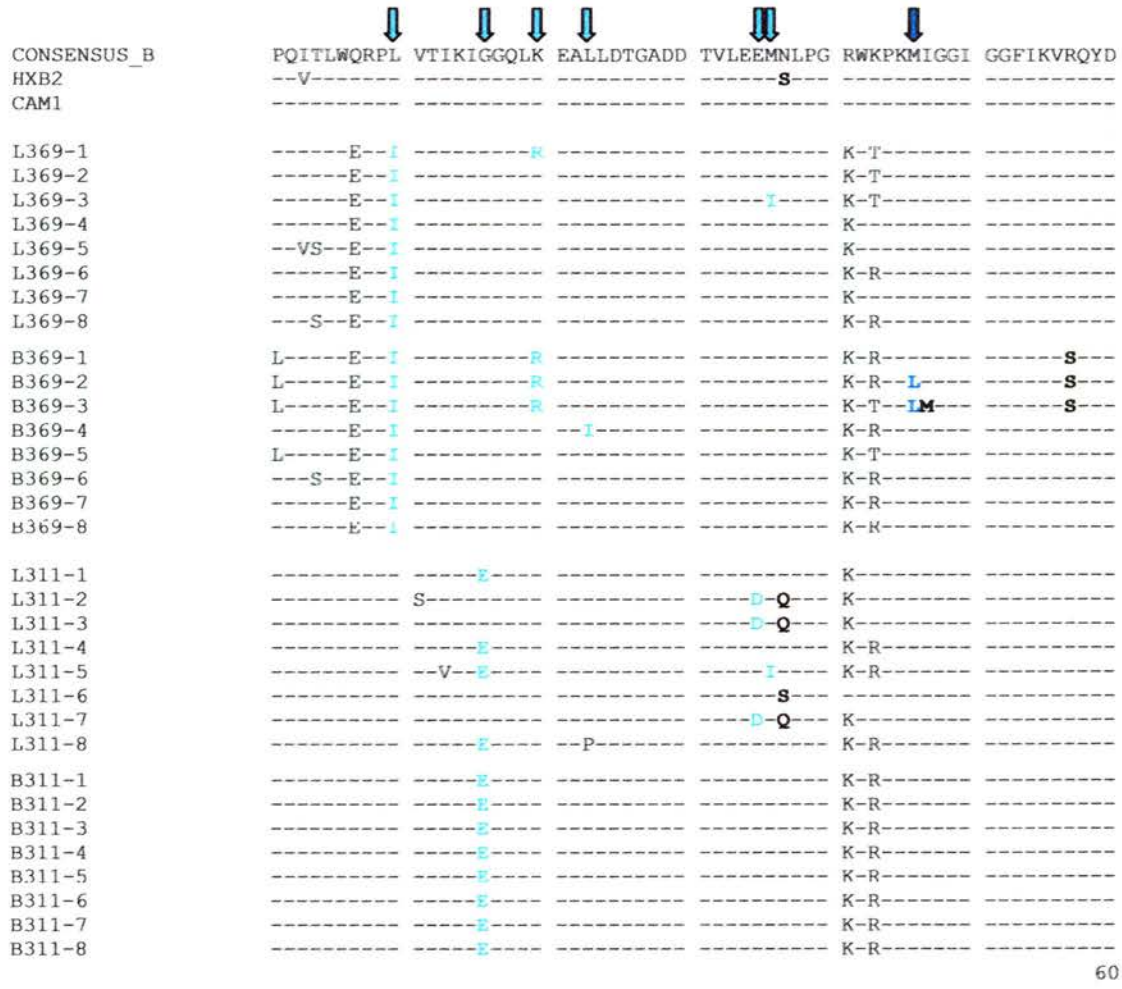


Figure 4.9: Predicted PR amino acid sequences of PCR products amplified at limiting dilution from post-mortem tissue. L: Lymphoid tissue derived; B: Brain tissue derived. Dashes indicate identical bases. Dots indicate an alignment gap. Stars indicate a stop codon. Minor protease inhibitor resistance mutations, indicated by light blue arrows, are L10I (Kempf et al., 2001; Watkins et al., 2003; Condra et al., 1996), G16E (Carrillo et al., 1998), K20R (Kempf et al, 2001; Molla et al., 1996; Condra et al, 1996), L23I(Croteau et al., 1997; Kempf et al, 2001; Watkins et al, 2003; Condra et al, 1996), E35D (Rusconi et al., 2000), M36I (Molla et al, 1996), L63P (Kempf et al, 2001; Watkins et al, 2003; Gong et al., 2000; Condra et al, 1996), A71T (Patick et al., 1995; Kempf et al, 2001; Markowitz et al., 1998; Condra et al, 1996; Rusconi et al, 2000) and A71V (Kempf et al, 2001; Markowitz et al., 1995; Markowitz et al, 1998; Croteau et al, 1997; Gong et al, 2000; Gatanaga et al., 2002; Partaledis et al., 1995; Watkins et al, 2003). The only major protease inhibitor resistance mutation, shown in dark blue, is M46L, (Kaplan et al., 1994; Kempf et al, 2001; Watkins et al, 2003; Molla et al, 1996; Croteau et al, 1997; Partaledis et al, 1995). All mutations confer resistance to multiply protease inhibitors.

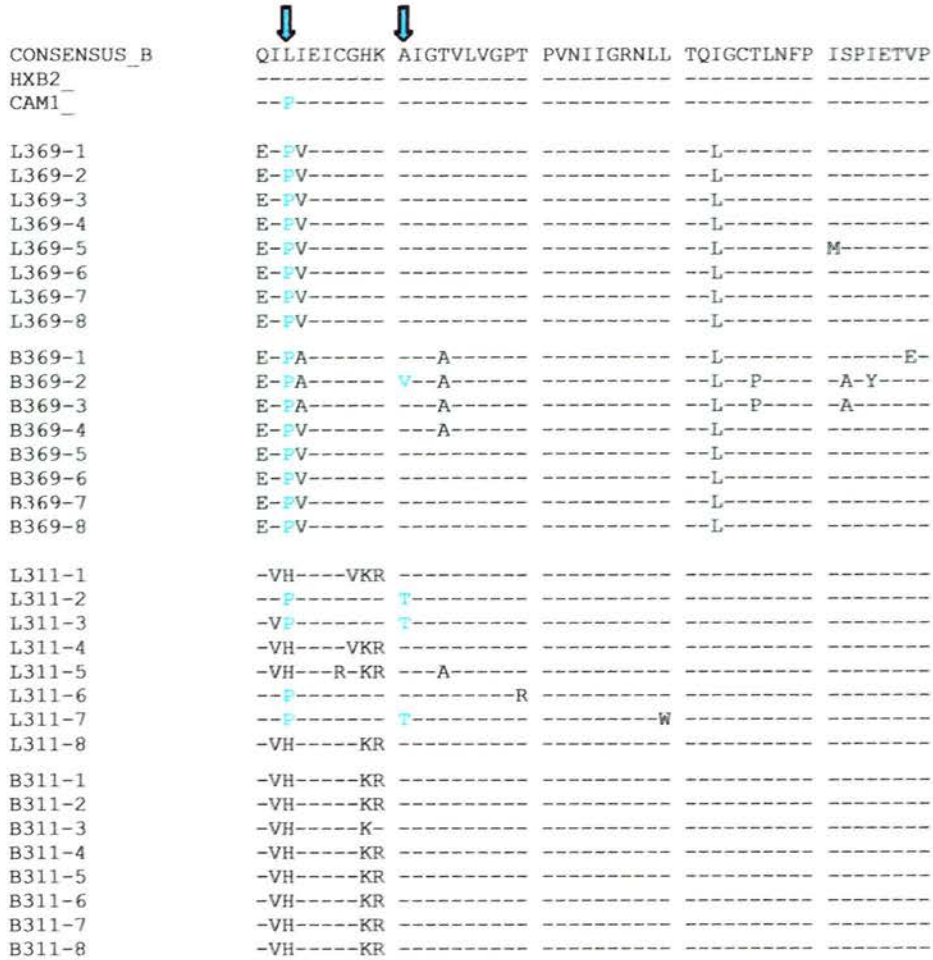


Figure 4.9: Continued.

Subject	Tissue	No	L10 →I	G16 →E	K20 →R	L23 →I	E35 →D	M36 →I	**M46 →L**	L63 →P	A71 →V	A71 →T
369	L	1	●		●					●		
		2	●							●		
		3	●					●		●		
		4	●							●		
		5	●							●		
		6	●							●		
		7	●							●		
		8	●							●		
	B	1	●		●					●		
		2	●		●				●	●	●	
		3	●		●				●	●		
		4	●			●				●		
		5	●							●		
		6	●							●		
		7	●							●		
		8	●							●		
311	L	1		●								
		2					●			●		●
		3					●			●		●
		4		●								
		5		●				●				
		6								●		
		7					●			●		●
		8		●								
	B	1		●								
		2		●								
		3		●								
		4		●								
		5		●								
		6		●								
		7		●								
		8		●								

Table 4.5: Summary of drug resistance mutations present in PR sequences amplified at limiting dilution from brain and lymphoid tissue. **Dark blue**: major mutation- confers intermediate resistance to some protease inhibitors. Light blue: minor mutations- may contribute to resistance (Rhee et al, 2003).

Unfortunately, after the sequencing work had been completed, it emerged that there had been a clerical error at the time of the post-mortems on these subjects. Further investigations of the clinical records and interviews with the clinicians responsible for the care of the individuals revealed that PIs had not in fact been prescribed to these subjects. The data has nevertheless been included here as it will serve as a valuable control for further investigations (now on-going) into the distribution of drug-resistance mutations in individuals who have taken PIs.

Only one mutation which may contribute an intermediate degree of PI resistance, M46L, was present in any of the sequences (B369-2 and B369-3). It is possible that this mutation was acquired through dual-infection and recombination with a drug-resistant variant from an individual taking a PI. Alternatively the variant which originally infected this subject may have harboured the M46L mutation. This has been described in a previous study although it appears to occur rarely; only one of 38 acutely infected subjects harboured virus possessing the M46L mutation (Re et al., 2004).

All other mutations detected in the PR sequences were minor resistance mutations which may contribute to resistance in the presence of other mutations but which are also present at a relatively high frequency in drug-naïve subjects (Re et al, 2004). Thus the presence of these drug resistance mutations is consistent with the clinical data showing that these individuals did not take a PI. In subject 311 these minor mutations appear to be overrepresented in the lymphoid-derived sequences while in subject 369 they are slightly overrepresented in the brain-derived sequences.

4.4 Discussion

In this study the pattern of drug-resistance associated mutations found in HIV proviral DNA isolated from post-mortem brain and lymphoid tissue were compared. Examination of RT sequences revealed that for three out of four study subjects, the drug-resistance associated mutation pattern was similar in both compartments. In one subject, there were significantly more drug-resistance associated mutations in lymphoid-tissue sequences than in those amplified from the brain.

A surprising finding was that sequences amplified from the two subjects who had died from AIDS-related illnesses had very few drug-resistance associated mutations. In both cases there was a period of some months (15 months for subject 320 and 6 months for subject 369) between the cessation of treatment and death. Studies have shown that almost all individuals taking one or two NRTIs only for at least a year, harbour virus containing drug resistance mutations (GomezCano, et al. 1998). Thus it is highly likely that at the time of receiving treatment, these individuals harboured virus which contained some degree of resistance to the NRTIs they were taking. It is probable that the rapid viral replication which presumably took place during the later stages of illness after treatment had been stopped, allowed drug-sensitive variants to expand and predominate. It would be expected that drug-resistant proviral copies would still be present in the DNA of long lived cells. However, explosive replication of drug-sensitive variants after the cessation of treatment may mean that the relatively small sample of sequences carried out in this project was not sufficient to detect such

"archived" copies.

It is interesting to note that there were also considerable intervals between the end of treatment and the death of the subjects who had not progressed to AIDS (11 months for subject 311 and 45 months for subject 234). Despite this, a large number of drug-resistance conferring mutations are present in the sequences derived from these subjects; particularly for subject 311. There have been reports of maintenance of drug-resistance mutations in the viral population despite no anti-retroviral drug for extended periods of time after transmission of drug-resistant variants (Delaugerre et al., 2004). It is thought that the maintenance of resistant variants in these circumstances is because there is no "reserve" of wild-type virus to outgrow other variant on cessation of therapy. Thus subject 311 may have been initially infected with drug-resistant variant. Additionally, viral replication in pre-symptomatic subjects may be more controlled by host immunity and thus evolution to wild-type virus may take longer. Some sequences amplified from this subject have few or no drug-resistance associated mutations suggesting that over time these variants may have become dominant.

If an organism reproduces asexually and recombination does not occur, the evolutionary relationships implied by phylogenetic analysis of sequences amplified from different regions of the genome should be identical. In contrast, comparison of the phylogenetic trees obtained in this study for different regions of the HIV genome from single study subjects reveals that there are discordant phylogenetic relationships. Most strikingly, the V3 sequences from subjects 369 and 234 segregate according to tissue of origin

yet the RT sequences from the same anatomical sites are intersperced. This suggests that in these cases a process of recombination has contributed to the observed genetic diversity. This is consistent with previous reports of frequent *in vivo* recombination events (Morris et al, 1999; Dykes et al., 2000). The packaging of two homologous copies of the RNA genome in each virion underlies this phenomenon. An alternative hypothesis is that the selective pressures on RT sequence are identical in the CNS as they are in the periphery while the selective pressure on V3 differs between the two sites. If this were the case then a process of convergent evolution could lead to the phylogenetic grouping of brain derived V3 sequences.

Recombination plays an important role in the global genetic diversity of HIV as demonstrated by the high prevalence of circulating recombinant forms (CRFs) in some areas (Gao et al., 1996; Gao et al., 1998). As yet there has been no evidence that recombinant forms arise and become dominant in a given region due to any selective advantage. Thus it has been assumed that the differing geographical distribution of CRFs is merely due to stochastic events. In contrast, the consistent findings of intra-patient recombinant forms clustering according to anatomical sites, strongly suggests that recombination is an important adaptive mechanism for HIV in response to selective pressures. One such selective pressure is the constraint on viral attachment and entry imposed by differential expression of host cell receptors. It is noteworthy that in this and previous studies (Morris et al, 1999) the genetic region which shows the greatest degree of segregation according to anatomical location is V3. This region is the major determinant of HIV

tropism and thus it appears that the presence of different cellular targets in different locations is a crucial selective pressure. Once HIV enters a new anatomical region, there will be selection of variants with mutations in V3 which are able to attach and infect cells more efficiently. Other genomic regions which do not face such a strong selective pressure in the new environment may maintain similar sequences to variants in other locations through a process of recombination. Thus differential selective pressure appears to play an important role in the compartmentalisation of V3 sequences observed between brain and lymphoid tissue.

Similar evidence of frequent recombination events between brain and lymphoid tissue derived sequences has led some investigators to suggest that genetic drift due to anatomical separation is not an important factor in the segregation of sequences according to location. However, examination of the sequences in this study reveals evidence of just such a process of genetic drift. A puzzling finding was that the RT sequences derived from brain and lymphoid tissue for study subject 320 are phylogenetically distinct despite no evidence for differences in the pattern of drug resistance mutations between these two compartments. Examination of the nucleotide sequences revealed that the split was mainly due to two synonymous mutations. It is difficult to envision how such mutations could provide any selective advantage to variants and thus this strongly suggests that a process of genetic drift and/or stochastic events have contributed to the divergence of brain and lymphoid tissue derived RT sequences in this subject. The infection of the brain may have been seeded by variants which were not representative of the entire

viral population present in the lymphoid system. Some degree of anatomical segregation may have then contributed to the separate evolution of the two lineages.

Examination of the PR nucleotide sequences reveals that there is differential distribution of some synonymous mutations between the lymphoid tissue and brain derived sequences for both subject 369 and 311 (data not shown). Thus it appears that genetic drift may also have played a role in the segregation of these lineages. However, non-synonymous mutations are also unevenly distributed resulting in different patterns of minor drug-resistance associated mutations between the two compartments. The mechanism underlying this phenomenon is unclear. It is possible that a combination of genetic drift and stochastic events have contributed. Alternatively it is possible that some selective pressure is directing differential evolution in the two compartments. Neither of these individuals appears to have taken a PI and thus differential drug-pressure is not likely to have played a role. However, it is possible that some subtle differences in cellular biochemistry between lymphoid and brain cells selects for certain mutations in the protease gene.

A hypothesis which is often mentioned in the scientific literature is that sub-optimal levels of anti-retroviral drugs may lead to evolution of drug-resistant variants in the CNS. This study found no evidence of such a phenomenon. In fact, investigation of one pre-symptomatic study subject revealed far more drug-resistance mutations in lymphoid-derived sequences than in those amplified from brain tissue. This results is in common with previous studies

which have identified cases with more drug-resistance mutations in the lymphoid system than in the brain but no cases where the opposite is true (Wong et al, 1997; McClernon et al, 2001). One explanation for the failure to support the hypothesis mentioned above is that the levels of drug found in the CNS may be far lower than many have previously assumed (see section 4.1 for more details). Thus, rather than there being sub-optimal levels of drugs allowing evolution of resistant variants, there may in fact be only negligible levels of drugs. This would allow the continued growth of drug-sensitive variants after the selective pressure of the drugs has removed them from the periphery.

This does not mean that the evolution of drug resistance-associated mutations does not occur in the brain. As mentioned previously, it is thought that the concentration of zidovudine at a given location in the brain is inversely correlated with the distance from the brain surface (Blasberg et al, 1977). Thus there may be a layer of the brain at which the drug concentration is sufficient to inhibit growth but not high enough to eliminate viral replication entirely. Such conditions would indeed promote the evolution of drug resistant variants. The method used in this project may be too crude to detect this process although it is possible that the lineage of brain-derived sequences amplified from subject 369 which possessed a high number of drug-resistance mutations could be due to such a process. The emergence of new techniques could lead to exciting advances in this area. For example, the use of in situ PCR or laser capture microdisection followed by PCR, would allow the occurrence of drug-resistance associated mutations at a

given location to be correlated with distance from the brain surface.

Chapter 5: Results III

5.1 Introduction

HIV associated dementia (HAD) affects approximately 30% of people with untreated AIDS (Mcarthur et al., 1993). Thus, given the huge number of individuals infected with HIV, HAD is likely to be the leading cause of dementia worldwide. The neuropathological changes associated with HAD are termed HIV encephalitis (HIVE). The pathognomic feature of HIVE is the presence of multinucleated giant cells (Budka et al., 1991). Other pathological features may include astrocytosis, myelin pallor, infiltration of monocytic cells and neuronal loss (Bell, 1998). Current opinion is that the observed pathology arises as a result of a cascade of damaging processes initiated by infected microglia and macrophages (reviewed in Kaul et al., 2001; Mollace et al., 2001). However, a vigorous debate continues as to the extent of infection of other resident brain cells and their potential contribution to neuropathology.

Conflicting results about the brain cells infected by HIV have emerged from immunohistochemical studies (reviewed in Brack-Werner, 1999). Most studies have stained cells with antibodies against HIV structural proteins which appear late in the viral life cycle. The most common peptide stained for is the capsid portion of the Gag polyprotein (p24 antigen). Such studies have detected viral proteins mainly in macrophages, giant cells and microglia (Vazeux et al., 1987; Nuovo et al., 1994) or occasionally also in astrocytes (Saito et al., 1994; Pumarola Sune et al., 1987). In contrast, early viral proteins such as Nef and Rev have been found frequently in astrocytes but rather less commonly in microglia/macrophages (Saito et al, 1994; Ranki et

al., 1995). This suggests that astrocytes are infected *in vivo* but have a very limited capacity to produce structural proteins and therefore infectious virus.

In situ hybridisation studies have generally identified viral RNA mainly in microglia/macrophages (Koenig et al., 1986). However, in keeping with the immunohistochemistry studies, viral RNA coding for early proteins has been detected in up to 20% of astrocytes (Saito et al, 1994; Ranki et al, 1995). The main limitation of in situ hybridization is that a large amount of RNA must be present to give a positive result. This means that results obtained using this technique may underestimate the number of infected cells. In particular cells in which low levels of viral RNA are produced, are unlikely to be detected as positive.

To overcome this problem, a technique called PCR amplified in situ hybridization (or simply in situ PCR) has been devised. In this technique, PCR amplification is carried out with the cells themselves acting as the vessels for the reaction (Bagasra et al., 1993). In common with other techniques, in situ PCR has demonstrated abundant infection of microglia. A number of studies have also reported low level infection of astrocytes or neurons (Bagasra et al., 1996; Nottet et al., 1996). Interpretation of these results is complicated by the frequent occurrence of false positives when using in situ PCR. Numerous factors can influence the likelihood of false positives arising including the number of PCR cycles, the length of incubation with proteinase K and the size of the PCR product (Haase et al., 1990; Strappe et al., 1998). False positives appear only on slides where some cells are truly positive. This suggests that they occur due to diffusion of PCR

product between cells. Thus the inclusion of HIV negative slides does not provide an adequate control. That the results of some in situ PCR studies might reflect a high degree of false positives is supported by the fact that the calculated proviral loads are often far higher than those found using tube based PCR systems (Bell et al., 1998).

Thus, despite the plethora of techniques available, the question of which brain cells are infected and to what extent remains controversial. One approach which could help resolve this would be to purify different brain cell types and then test each population separately and directly for HIV infection using PCR.

Separation of individual brain cell types has posed a major challenge to scientists for many years (reviewed in Poduslo and Norton, 1975). The cellular structure of neural tissue is immensely complex with most cell types possessing some form of cellular processes which connect and intertwine with other cells and cellular processes (reviewed in Berry et al., 2002). Neurons have two types of process which allow the propagation of electrical signals; dendrites receive afferent signals while axons deliver efferent signals to adjacent neurons. Projections from oligodendrocyte cellular membranes form myelin sheaths which wrap around neuronal axons forming concentric lamellae. The multiple projections emerging from astrocytes give them their characteristic "star-like" shape. These processes can connect with other astrocyte processes to allow signal propagation. In addition they can ensheath blood capillaries contributing to the blood brain barrier or connect with the nodes between myelin sheaths and thus influence neuronal

signalling. Microglia also possess processes and in this respect are unique amongst known tissue macrophages. All these connections mean that brain cells are tightly lashed together and thus separation is extremely difficult.

The first stage in any technique for bulk separation of brain cells is to form a single cell suspension. This usually involves some type of enzymatic digestion, followed by mechanical agitation to tease out cells followed by forcing the cell mixture through some type of mesh to ensure that only single cells are present. The exact method employed depends on the subsequent application. For example if the morphology of cells is to be examined, very gentle techniques of cellular disaggregation must be employed. If cell surface antigens are to be examined (or used to aid cell separation as in FACS sorting) then the enzyme used must be carefully chosen to avoid digestion of the antigen of interest.

The most effective way to obtain large populations of "pure" brain cells is to culture primary brain cells using conditions which selectively inhibit growth of all cell types other than the one of interest. This approach has been successfully used to isolate oligodendrocytes, astrocytes, microglia and neurons (Shi et al., 1998; Budziszewska et al., 2002; Liuzzi et al., 2004). The major disadvantage of this method is that the cells undergo change and differentiation *ex vivo*. This means that the phenotype of cells obtained may not accurately reflect that found *in vivo*.

For many years the only method available for separation of cells from a cell suspension without culturing was isopycnic separation using solutions of various densities. Various media can be used to form density gradients

including sucrose, albumin, Ficoll and Percoll. There are two types of density gradient: continuous or discontinuous (figure 5.1). A continuous gradient is formed when a tube containing media of a given dilution is centrifuged at a high g force. This results in a gradient of density being formed with the highest density being at the base of the tube and the lowest at the top. Cells to be separated can be layered on top of the gradient and the tube centrifuged at a low g force. The cells or cellular components will move down the gradient and settle where the density of the media equals their own density. As cells of a given type will have a range of densities, bands of cells in a continuous gradient will be wide and can be difficult to visualise.

To make a discontinuous gradient, various dilutions of media are prepared. These are layered in a tube starting with the most dense and working up to the least. Layering must be carried out very carefully so that the interfaces between layers are distinct. The cells to be separated are layered on top of the gradient. The tube is then centrifuged at a low g force. Cells descend through the gradient and settle at an interface where the layer above is less dense and the layer below is more dense than the cell itself. Cells with a range of densities (i.e. the range between the densities of the two adjacent layers) will form single narrow bands. Thus this method will produce more discreet easily visible bands.

Methods based on tissue disaggregation followed by density gradient separation have been used in many studies for separation of cell types with various degrees of success (reviewed in Poduslo and Norton, 1975). The major problem with this approach is the heterogeneity of brain cells of a given

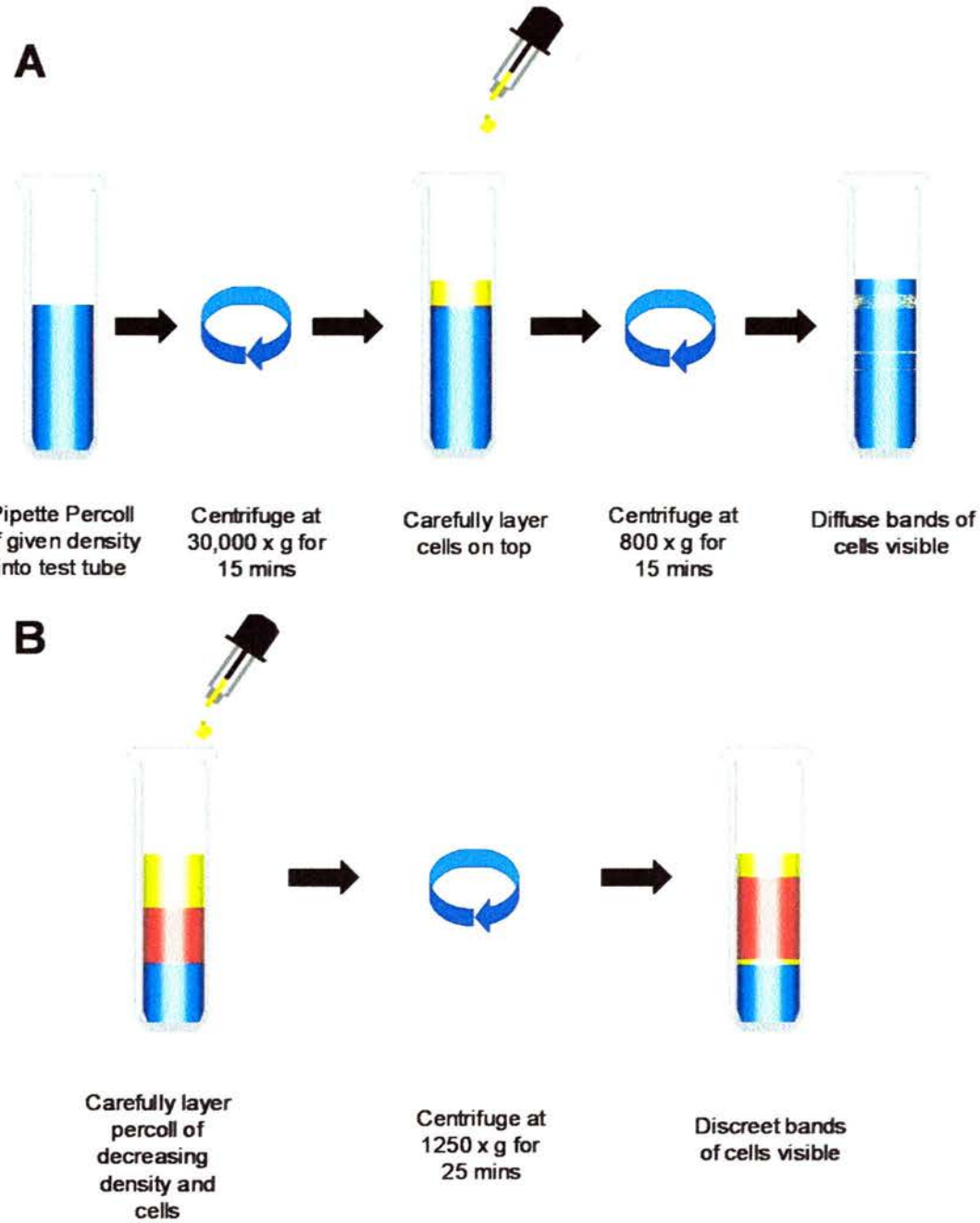


Figure 5.1: Schematic representation of separation of cells using Percoll gradients. A: Separation using a continuous gradient results in diffuse poorly visible bands of cells. Each band contains cells within a narrow range of densities. B: Separation using a discontinuous gradient results in clearly defined bands of cells. Each band contains cells with a wider range of densities.

type. The ranges of densities characteristic of given cell types overlap and thus it is very difficult to obtain pure populations using this method. A method which attempted to overcome this problem used an initial separation using differential centrifugation in Ficoll media (Farooq and Norton, 1978). This allowed enrichment of either astrocytes or neurons on account of their differences in cell size and therefore sedimentation rates. These enriched fractions were then separated using a discontinuous Ficoll gradient. Using this method, neurons of 90% purity and astrocytes of 57% purity were obtained.

While different cell types have quite similar and overlapping ranges of densities, the broken processes and cell debris produced by tissue homogenisation are distinctly less dense than whole cells. This is because cell processes in general are composed mainly of lipid membrane with relatively little cytoplasmic content. Thus density centrifugation can very effectively separate whole cells from cellular debris.

Recently, various studies have attempted to purify brain cells using fluorescence activated cell sorting (FACS). FACS sorting allows separation of cells according to differences in size, density and antigen expression. This technique has been largely used for analysis of blood cells which are found in a preformed single cell suspension and are more or less devoid of cellular processes (reviewed in Kantor and Roederer, 1997). Clearly, to separate brain cells a single cell suspension must first be obtained. In addition, it is necessary to remove the cellular debris and cellular processes which constitute the bulk of the matter in a crude brain cell suspension prior to

FACS analysis in order to prevent clogging of the machine (personal observation). Despite the inherent difficulties, studies have been published recently which use FACS either for analysis of cell surface antigens or for cell sorting of brain cells (Malatesta et al., 2000; Morelli et al., 1999; Sedgwick et al., 1991).

It should be noted that most previous studies have used brains from mice or rats which have been obtained under optimum conditions. The brains were removed immediately following the death of the animal and in some cases blood contamination was eliminated by prior infusion with saline solution. Separation of human brain cells from post mortem tissue is complicated by a number of factors. Firstly, the interval between death and refrigeration can be many hours. The post mortem interval is often one to two days and can be much longer. Finally, transport of the brain from the mortuary to the laboratory can take up to a day. Thus the tissue has had ample time for significant cell lysis and decay to occur compared with laboratory animal studies where the brain can be removed and experimental work started immediately following death.

5.2 Aims

The primary aim of this study was to develop a technique for bulk isolation of pure cell fractions from post mortem human brains.

A secondary aim was to use this technique to separate brain cells from HIV infected human brains and assess the distribution and proviral loads of HIV in different cell types.

5.3 Results

A log of all cell separation experiments carried out is provided in Appendix II. The following text (sections 5.3.1 to 5.3.5) provides a more detailed explanation of the optimisation of various aspects of the cell separation procedure.

5.3.1 Tissue disaggregation

Initially, digestion was carried out using a previously published method involving papain and collagenase (Wright et al., 1997) (section 2.4.3). This method produced a single cell suspension although cellular morphology was not preserved. Attempts to separate cells using ficoll enrichment followed by a continuous Percoll gradient were preceded with a controlled trypsin digestion (Chatterjee and Sarkar, 1984b) (section 2.4.4). This method produced a cell suspension where cellular morphology was well preserved. This allowed identification of cell types based on a combination of immunostaining and cellular morphology. Unfortunately, cells prepared in this manner did appear to be particularly "sticky". This could be due to the preservation of cellular processes. Thus this method was replaced with a standard method of trypsin digestion (section 2.4.5) for subsequent FACS analysis. This technique produced a single cell suspension however there was a concern that trypsin may be causing cell death and thus decreasing cell yields. For this reason, a novel method of cell digestion was adopted which involved purified collagenase and DNAse (Ford et al., 1995) (section 2.4.6). It was found using mouse brains that the cell yields obtained were higher using this method than when trypsin digestion was used (data not

shown).

5.3.2 Density gradients

The first method of cell separation to be investigated was isopycnic separation using a continuous Percoll density gradient. Prior to using a continuous Percoll gradient, it is necessary to produce a calibration curve. This allows the selection of the optimum concentration of Percoll for the separation of the cells of interest. Due to variations in batches of Percoll, centrifuges and local conditions, this may differ from lab to lab. To produce the calibration curve, preformed Percoll gradients were produced using various different concentrations of Percoll. Coloured beads of known densities were separated using these gradients in the same manner as a cell suspension. The distances travelled by beads of different densities in each of the gradients were noted and a calibration curve created (figure 5.2).

The densities of different cell types as reported by previous studies are shown in table 5.1. Using the calibration curve, it was decided that a gradient using between 20 and 60% (v/v) percoll would give the optimum separation of cells within the predicted range of densities. Separation of brain cells was attempted using continuous Percoll gradients of a range of concentrations. The results of these attempts were that a thick band of material formed near the top of the gradient. This band had a predicted density of approximately 1.022g/ml. No other bands of cells were visible. The thick band of material was examined microscopically and determined to be largely composed of cellular debris and fragments of myelin. Some cells were also present in this fraction. As brain cells are predicted to have a higher density than 1.022g/ml,

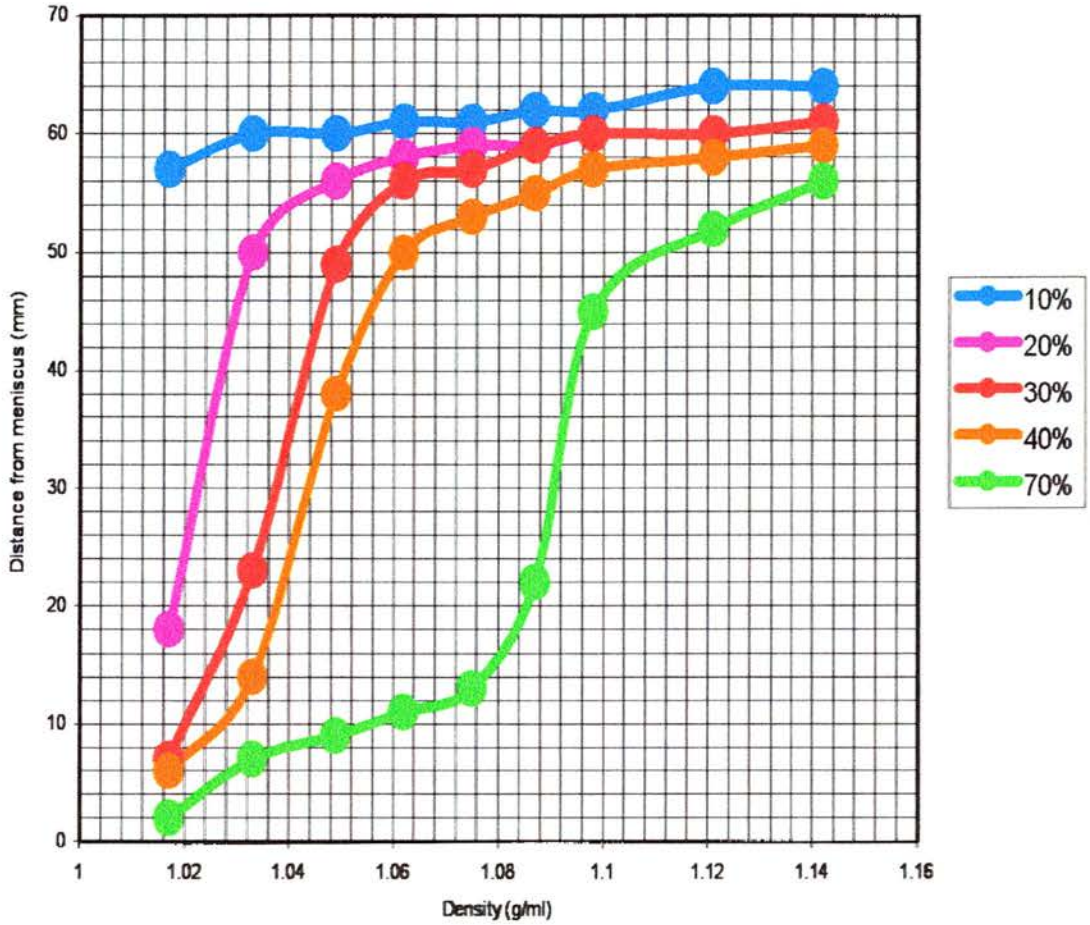


Figure 5.2: Calibration curve showing distance migrated in preformed Percoll gradients against density. Experiments were carried out using commercially available density marker beads using different percentage compositions of Percoll.

Cell type	Species	Density (g/ml)	Reference
Astrocytes	Mouse	1-1.045	(Wechsler-Reya and Scott, 1999)
	Rat	0.98-0.998	(Chatterjee and Sarkar, 1984)
	Rat	1.03-1.072	(Sedgwick et al., 1991)
Neurons	Mouse	1.045-1.085	(Wechsler-Reya and Scott, 1999)
	Rat	1.026-1.150	Chatterjee and Sarkar 1984
Microglia	Rat	1.03-1.072	(Sedgwick et al, 1991)
Leukocytes	Rat	1.072-1.088	(Sedgwick et al, 1991)
Myelin	Rhesus macaque	1-1.039	(Westmoreland et al., 2002)
	Rat	<1.03	(Sedgwick et al, 1991)
All Glia	Rhesus macaque	>1.039	(Westmoreland et al, 2002)

Table 5.1: Observed densities of brain derived cells from various species according to previous publications.

it was assumed that the cells were being "caught" in this layer due to clumping with lower density material. Attempts were made to reduce the propensity of the material to clump. First 2 mM EDTA was added to all media used in the preparation of a single cell suspension. This had little effect on cell clumping. Next the media used for storage of the brain tissue prior to separation was investigated. Brain material was stored in PBS, media A (Poduslo and Norton, 1975) or without media for 1 hour or 24 hours. Separation was carried out using a discontinuous Percoll gradient. Media A appeared to reduce the amount of clumping slightly; material stored in this media produced faint bands of cells following continuous Percoll separation at an approximate density of 1.026g/ml.

Concurrently, a method of cell enrichment using Ficoll media was investigated. This was an adaptation of a previously published method which had been used to isolate neurons and astrocytes from rat brains (Farooq and Norton, 1978). Ficoll enrichment was used prior to separation on a continuous Percoll gradient. A gentle trypsin digestion which aimed to maintain cellular morphology was used (Chatterjee and Sarkar, 1984a). This means that the morphology of cells, along with immunocytochemistry, could be used to identify cell types and thus calculate purities obtained. Ficoll media was used to produce pellets enriched for either neurons or astrocytes. Figure 5.3 shows that this method was able to enrich for these cell types and that cellular morphology was strikingly well preserved. However, the astrocyte enriched pellet contained a large amount of broken processes and cellular debris.

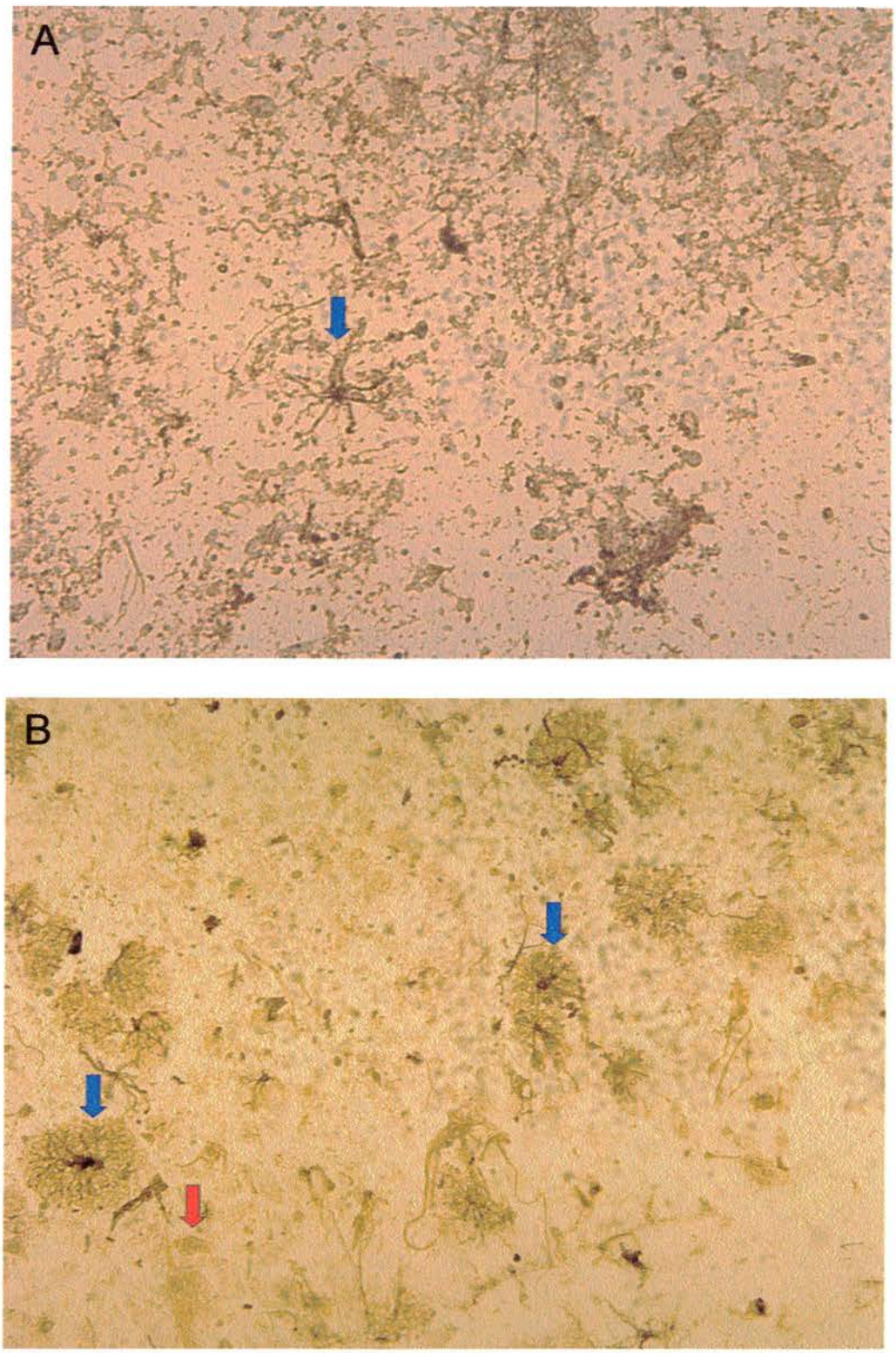


Figure 5.3: Immunocytochemical staining for GFAP (DAB with haemotoxylin counterstain) in cell fractions enriched for A: Astrocytes and B:.Neurons Visualised at 10 x magnification. Blue arrows indicate astrocytes. The red arrow indicates a probable neuron.

The next step in the published method was separation of cells from the Ficoll enriched pellets on a continuous gradient. The Ficoll enriched pellets were further separated using a preformed percoll gradient (40% (v/v) dilution). As previously a thick band of material with a density of approximately 1.022g/ml was apparent. A very faint band was visible lower down the tube. The estimated density of this band was 1.026g/ml. Microscopic examination of the top band revealed that it contained mainly cellular debris and broken processes however there were also a large number of cells present (data not shown). It appeared that the intact morphology of the cells caused them to be "caught" in the large amount of low density debris. It was not possible to obtain sufficient cells from the lower band to examine microscopically.

It was decided to use a more harsh digestion technique. This would result in loss of cellular processes which should make the cells less "sticky". In addition a discontinuous gradient was used so that bands of cells would be more easily visible. Previous reports using discontinuous percoll gradients had not been able to obtain pure cell fractions. However, removal of cellular debris had been achieved. Thus the discontinuous percoll gradient was used with the aim of separating cells from cellular debris. The cell suspension obtained could be used for alternative separation techniques.

A discontinuous gradient with layers of 60% and 30% (v/v) was prepared. Cells were suspended in PBS and carefully overlayed. Following centrifugation, a thick band was visible between the PBS and 30% layer. A thin band was visible between the 30% and 60% layers. Matter from both layers was aspirated and visualised microscopically (figure 5.4).

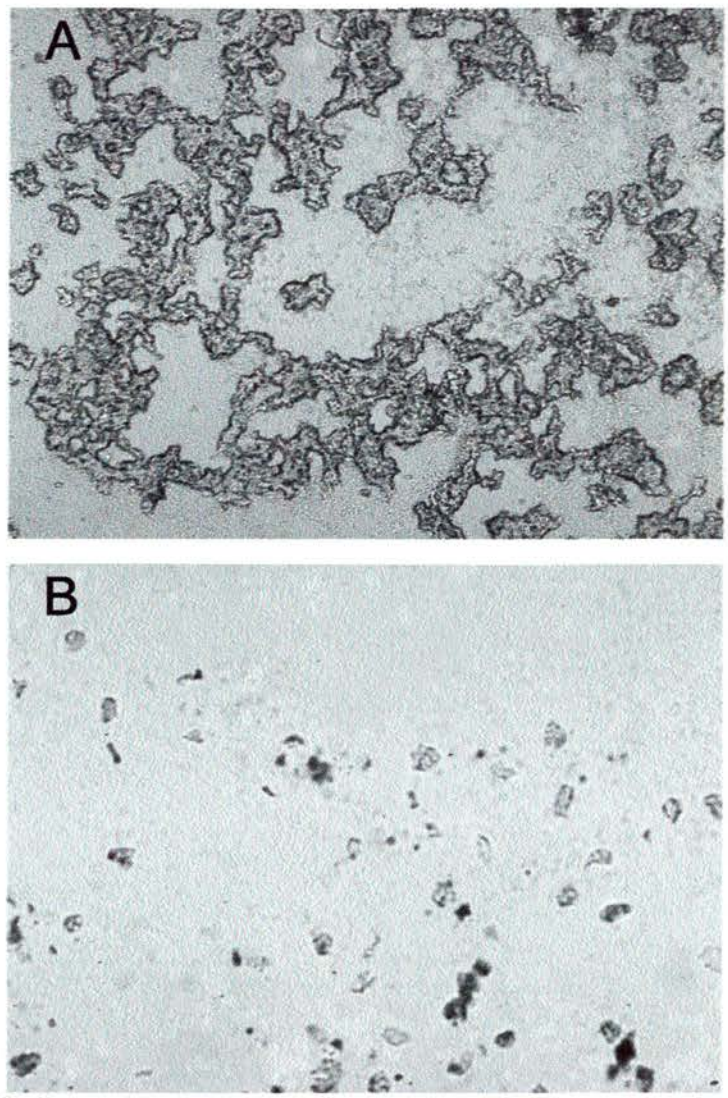


Figure 5.4: Cells and cellular debris aspirated from the interfaces of layers of a discontinuous percoll gradient viewed at 20 x magnification. A: Cellular debris and myelin fragments aspirated from above the 30% percoll layer. B: Cells aspirated from below the 30% percoll layer.

This technique effectively removes a large amount of cellular debris and thus was used prior to any FACS staining and analysis of brain cells.

5.3.3 Immunomagnetic separation

Immunomagnetic separation of glial cells has been successfully carried out previously using the Dynabead (Dynal Biotech) system (Wright et al, 1997). In our department immunomagnetic separation of blood cells has been routinely carried out using MACS beads (Milteny Biotech) and thus it was decided to attempt separation of brain cells with this system. MACS beads are considerably smaller than Dynabeads. Additionally, Dynabead stained cells are pulled to the side of an open test tube using a magnet whereas MACS bead stained cells become attached to a magnetic matrix within a column (figure 5.5).

Cells were incubated with anti-GAP43 or anti-CD45 antibodies and then Goat anti-mouse IgG MACS beads were attached. The stained cell suspension was passed through an automated MACS sorting machine.

Surprisingly, very little material emerged as a negative fraction and almost all cells emerged as the positive fraction for each antibody. It appears that due to the highly sticky nature of brain cells, all cells and cellular debris were becoming stuck to the true positive cells which were attached via beads to the magnetic matrix. Depletion of "sticky" cellular debris using a Ficoll gradient prior to MACS separation was attempted. Unfortunately this had little effect and most material continued to emerge as the positive fraction. Significantly, even in negative control separations where no antibody or beads were used, approximately 25% of the cell suspension emerged as the

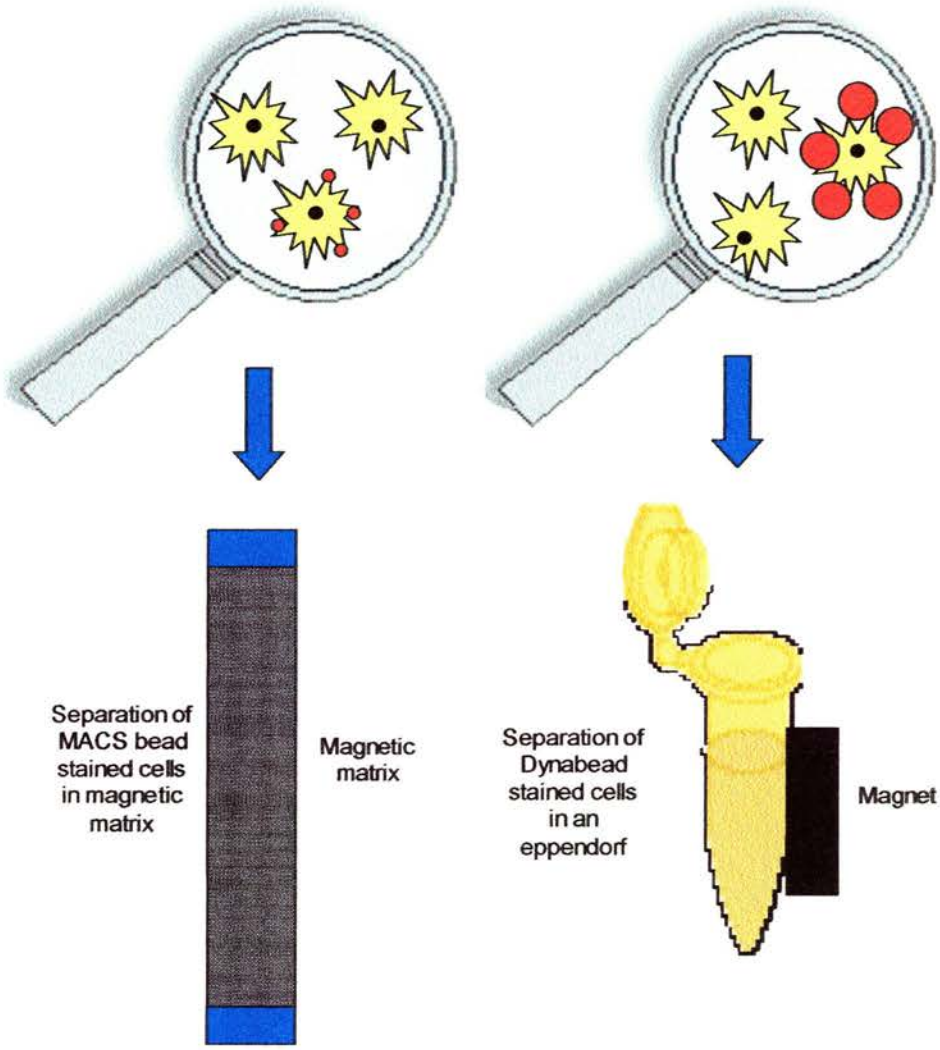


Figure 5.5: Schematic representation of two different methods of immunomagnetic separation using commercially available beads. The left-hand diagram shows separation using MACS beads while the right-hand diagram shows separation using Dynabeads. Note that MACS beads are much smaller than Dynabeads and that MACS bead separation involves passing the cells through a magnetic matrix while Dynabead separation is carried out in an eppendorf tube. This diagram is not drawn to scale; while the size of the Dyna beads relative to cells is similar to that shown, the MACS beads are in fact not visible even under 20 X magnification.

"positive" fraction. This suggests that cells were becoming lodged in the matrix even in the absence of magnetic bead staining and emerged only after repeated "flushing out". It was thus decided that MACS bead separation was not a viable option for separation of brain cells.

It seems likely that the use of a magnetic matrix during MACS bead separation explains why this technique was not successful while Dynabead separation has been previously reported. Indeed, initial attempts at separation using Dynabeads appeared to select a population of cells from the cell suspension. Unfortunately, immunostaining of these cells was inconclusive (data not shown) perhaps due to masking of antigens by other antibodies.

This line of investigation was discontinued due to promising initial results from FACS sorting of cells.

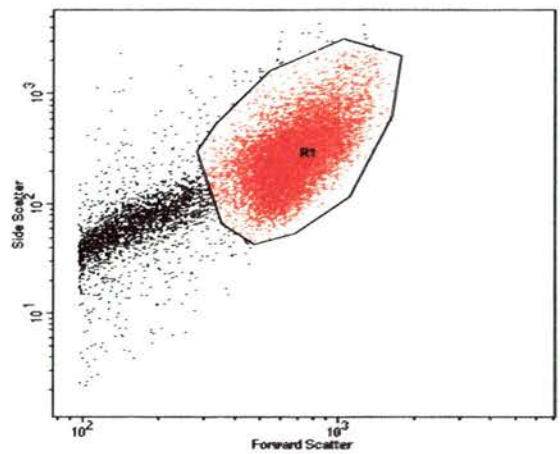
5.3.4 Cell fixation

HIV infected cells must be fixed prior to FACS analysis for biological safety reasons. In addition, if antibodies to intra-cellular markers are used, cells must be permeabilised. Standard fixation techniques make use of paraformaldehyde. Thus for initial attempts at FACS analysis, fixation in solutions of between 1 and 4% (w/v) paraformaldehyde was used. For the staining of intra-cellular markers subsequent permeabilisation using Triton solution was carried out. Unfortunately use of paraformaldehyde for fixation can adversely affect the DNA and is therefore not suitable if DNA extraction and PCR is to be carried out using the sorted cells. Ethanol fixation does not

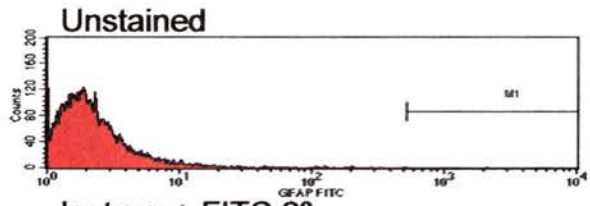
appear to damage nucleic acid (Gillespie et al., 2002; Ben Ezra et al., 1991). Fixation in ethanol has the additional advantage of permeabilising cells so no additional step is required. Initial attempts to fix cells in ethanol resulted in a great deal of cell clumping. This problem was overcome by using a modified "gentle" ethanol fixation technique (William Telford et al., 1997).

5.3.5 Flow cytometry

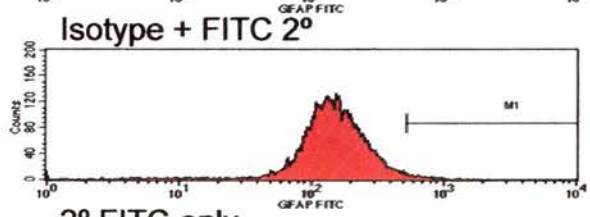
The antigen generally used to identify astrocytes is glial fibrillary acid protein (GFAP). GFAP is the main intermediate filament component of the astrocytic cytoskeleton (Eng et al., 2000). A monoclonal anti-GFAP antibody and a secondary antibody conjugated to FITC were used (n.b. Details of all antibodies are given in table 2.4). As GFAP is an intra-cellular antigen, all cells were fixed and permeabilised before staining. As mentioned previously, initial experiments used fixation with paraformaldehyde followed by Triton permeabilisation but eventually a gentle ethanol fixation procedure was adopted. The antibody was first tested on the human glioma cell lines U373 and A172. Expression of GFAP had been previously demonstrated in these cell lines using Western Blot analysis (Tlhyama et al., 1993; Khalid et al., 1999). The A172 were only weakly positive for GFAP (data not shown). The reason for this is not known but it is possible that *in vitro* passage of the cell line had altered its phenotype. As shown in figure 5.6, U373 cells were strongly positive for the GFAP antigen by FACS analysis. 45% of anti-GFAP stained cells fell within the marker M1 compared with only 1% of isotype control stained cells. However, the isotype control stained cells were far more fluorescent than unstained cells. Staining with the secondary antibody alone



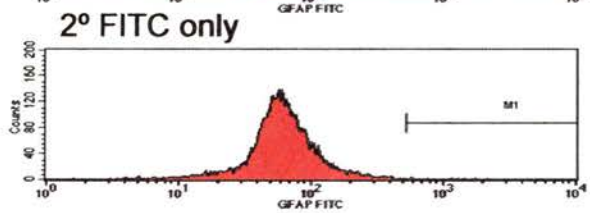
Region	Events	% Total
R1	13365	84.45



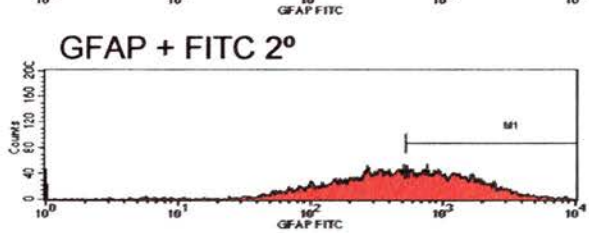
Marker	Events	% Gated	% Total
All	13365	100.00	84.45
M1	1	0.01	0.01



Marker	Events	% Gated	% Total
All	14568	100.00	84.67
M1	154	1.06	0.90



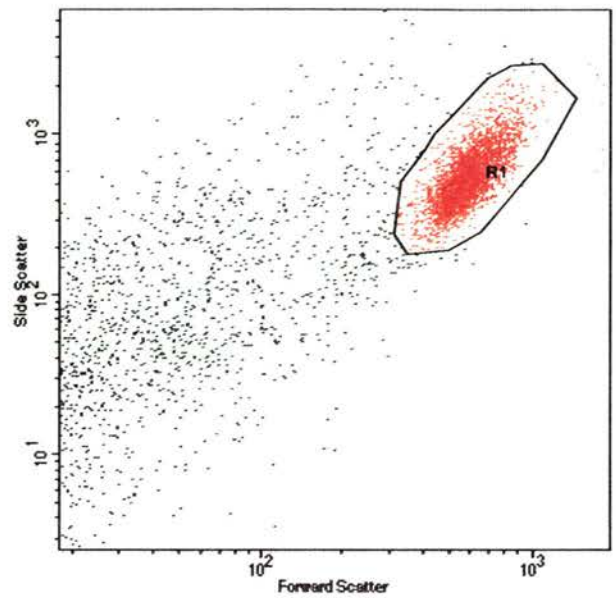
Marker	Events	% Gated	% Total
All	13016	100.00	86.77
M1	11	0.08	0.07



Marker	Events	% Gated	% Total
All	12424	100.00	82.83
M1	5901	47.50	39.34

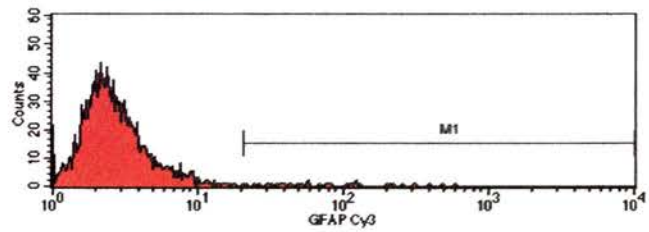
Figure 5.6: Flow cytometric analysis of astrocyte cell line (U373MG) stained with primary antibody and FITC conjugated secondary. Analysis of fluorescence was carried out on events which lay within the R1 gate as defined on the side versus forward scatter dot plot. Events lying out with this gate were presumed to be dead cells or cellular debris. On the histogram plots of or cells stained with the isotype control it can be seen that approximately 1% of cells lie within the M1 marker. On the histogram plot of cells stained with GFAP and a FITC conjugated secondary antibody approximately 45% of events lie within the M1 marker. Note than staining cells with either the isotype control plus a FITC conjugated secondary antibody or with the secondary antibody alone results in a significant increase in fluorescence compared to unstained cells.

revealed a high degree of non-specific staining. This is probably due to the permeabilisation of the brain cells. To circumvent this problem, an anti-GFAP antibody directly conjugated to Cy3 was obtained. As shown in figure 5.7, this antibody could be used successfully to stain the U373MG cells. In this case, the isotype control stained cells were not significantly more fluorescent than unstained cells. The antibody was next used to stain a single cell suspension of murine brain cells. The results of this are shown in figure 5.8. Two distinct populations were defined on the scatter profile. The proportions of anti-GFAP stained cells lying within the M1 marker were 86% and 83% for gates 1 and 2 respectively. This is compared to less than 1% of isotype-control stained cells. Thus it appears that astrocytes are present in both regions of the scatter plot. The antibody was used to stain a single cell suspension of human brain cells (figure 5.9). Histogram plots did not reveal a significant shift in fluorescence when anti-GFAP stained cells were compared to isotype control stained cells (data not shown). However a dot plot of fluorescence against side scatter revealed that a new population arises in the anti-GFAP stained cells. 58% of anti-GFAP stained cells fall within gate R2 compared to 8% of isotype control stained cells. Unfortunately, when this experiment was repeated using cells which had been stained and stored for a day, no such change in fluorescence was apparent (figure 5.10). This is probably due to the use of the fluorochrome Cy3. This fluorochrome has an optimum excitation wavelength of around 550 nm. This is higher than the wavelength used by the FACS machine available and thus the fluorescence is probably sub-optimal. It appears that storing cells stained with this antibody, even for short



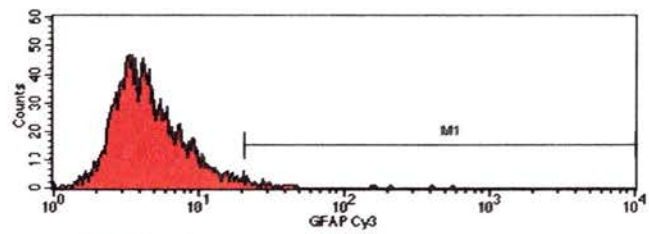
Region	Events	% Total
R1	3528	70.56

Unstained



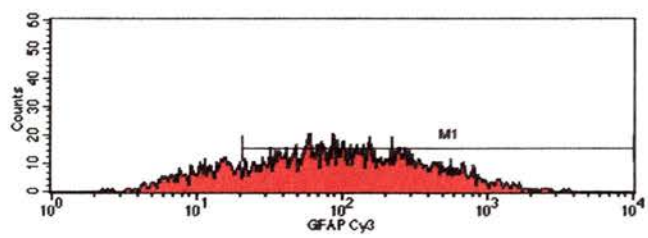
Marker	Events	% Gated	% Total
All	3528	100.00	70.56
M1	42	1.19	0.84

Isotype Cy3



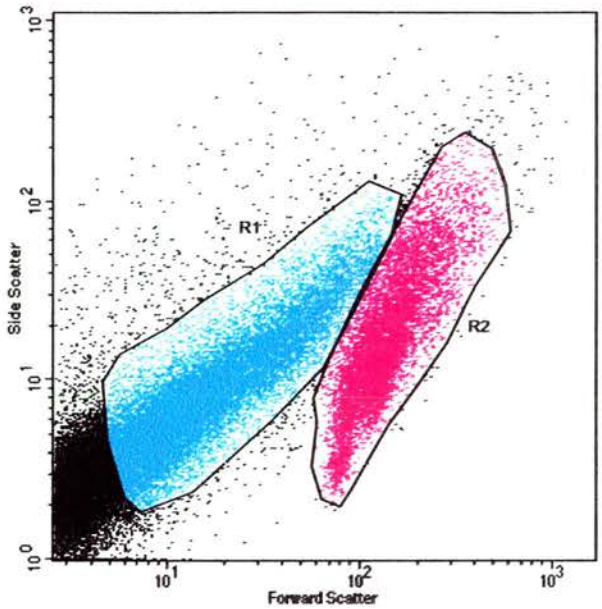
Marker	Events	% Gated	% Total
All	4479	100.00	89.58
M1	51	1.14	1.02

GFAP Cy3



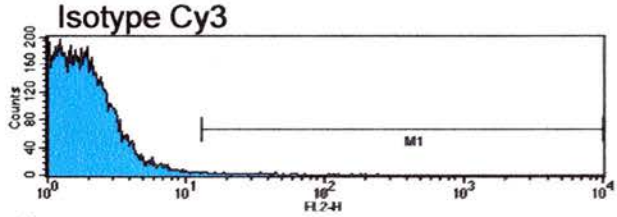
Marker	Events	% Gated	% Total
All	4441	100.00	88.82
M1	3674	82.73	73.48

Figure 5.7: Flow cytometric analysis of astrocyte cell line (U373MG) stained with directly conjugated antibody. The gate was defined as previously. On the histogram plots of cells stained with the isotype control it can be seen that approximately 1% of cells lie within the M1 marker. Note that there is not an increase of fluorescence using the isotype control compared to unstained cells. On the histogram plot of cells stained with the GFAP:Cy3 antibody approximately 83% of events lie within the M1 marker.

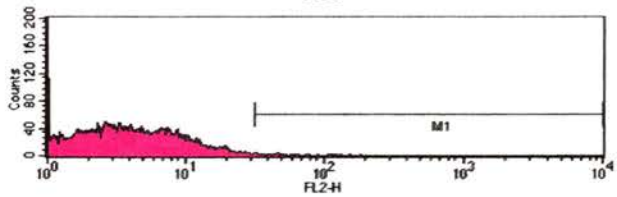


Region	Events	% Total
R1	30004	54.80
R2	9099	16.62

Isotype Cy3

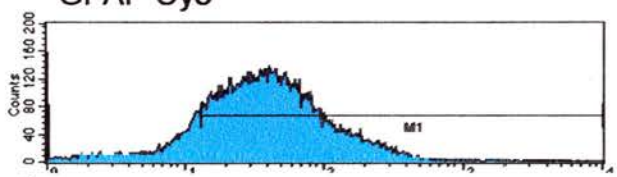


Marker	Events	% Gated	% Total
All	30004	100.00	54.80
M1	132	0.44	0.24

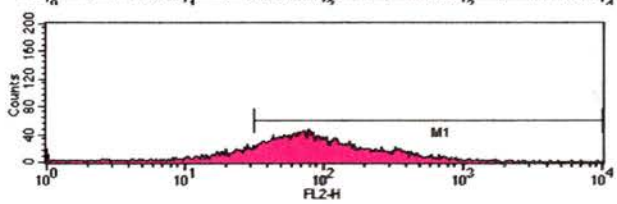


Marker	Events	% Gated	% Total
All	9099	100.00	16.62
M1	60	0.66	0.11

GFAP Cy3

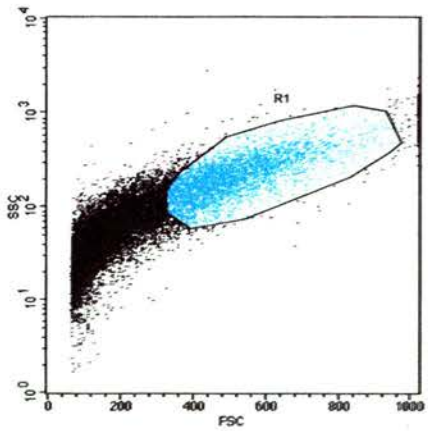


Marker	Events	% Gated	% Total
All	30619	100.00	61.24
M1	26234	85.68	52.47



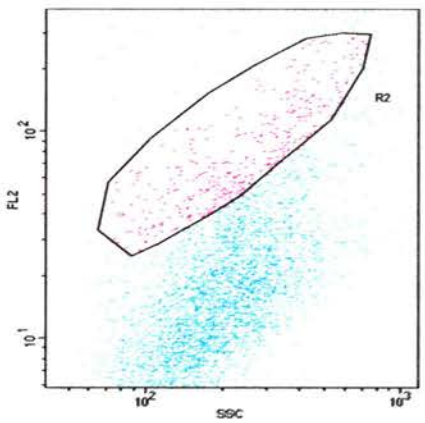
Marker	Events	% Gated	% Total
All	8178	100.00	16.36
M1	6778	82.88	13.56

Figure 5.8: Flow cytometric analysis of mouse brain cells stained with directly conjugated antibody. Two populations of cells could be seen on the scatter plot of side versus forward scatter. These were selected as separate gates in order to assess which contained GFAP positive cells. On the histogram plots of cells stained with the isotype control it can be seen that less than 1% of cells lie within the M1 marker. On the histogram plot of cells stained with the GFAP:Cy3 antibody approximately 85% and 82% of events lie within the M1 marker when events from within Gates R1 or R2 respectively are analysed.



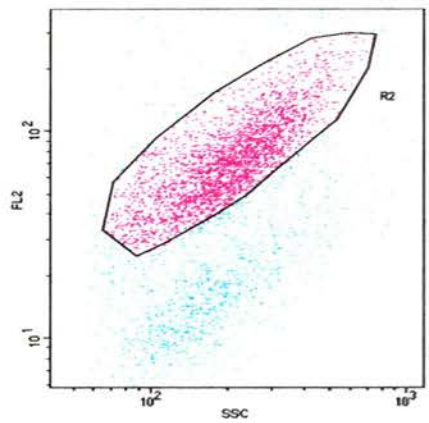
Region	Events	% Total
R1	4860	24.30
R2	478	2.39

Unstained



Region	Events	% Gated	% Total
R1	4860	100.00	24.30
R2	408	8.40	2.04

GFAP Cy3



Region	Events	% Gated	% Total
R1	4618	100.00	23.09
R2	2665	57.71	13.33

Figure 5.9: Flow cytometric analysis of human brain cells stained with directly conjugated antibody GFAP:Cy3. No distinct populations of cells were apparent from the dot plot of side versus forward scatter. Events chosen for analysis were defined by gate R1. It was assumed that most events falling outwith this gate were dead cells or cellular debris. Histogram plots comparing events within R1 showed little increase in fluorescence when cells stained with GFAP:Cy3 were compared with those stained with the isotype control (data not shown). However, a dot plot of fluorescence against side scatter, revealed that a new population arises in the GFAP:Cy3 stained cells. This population was defined on the dot plot by gate R2. When cells stained with the isotype control are analysed 8.4% of events fall with this gate. When cells stained with GFAP:Cy3 are analysed this percentage rises to 57.7%

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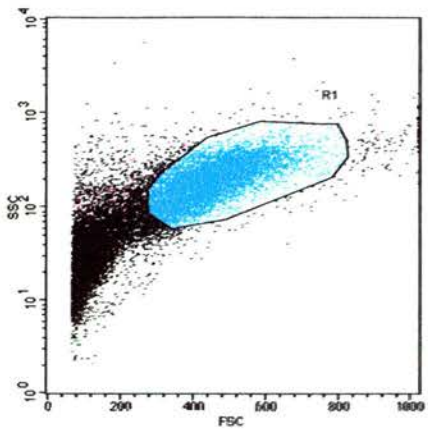
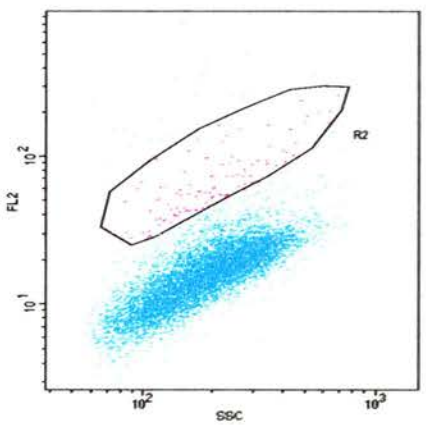


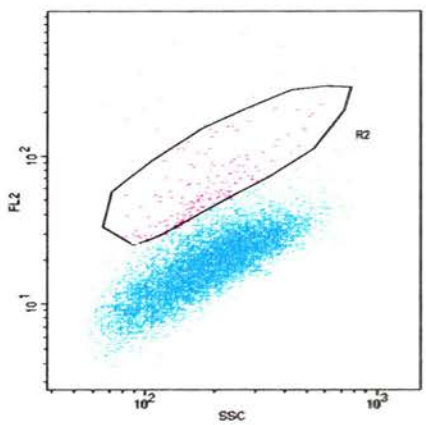
Region	Events	% Total
R1	10696	53.48
R2	255	1.28

Unstained



Region	Events	% Gated	% Total
R1	10696	100.00	53.48
R2	182	1.70	0.91

GFAP Cy3

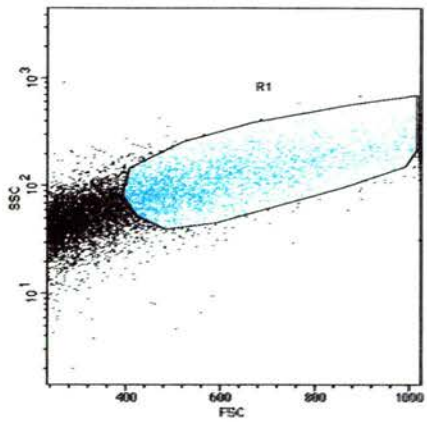


Region	Events	% Gated	% Total
R1	10416	100.00	52.08
R2	113	1.08	0.57

Figure 5.10: Flow cytometric analysis of human brain cells stained with directly conjugated antibody GFAP:Cy3 where stained cells have been stored for a number of days. Gate R1 was defined as previously. Unlike the previous experiment, there does not appear to be a significant new population when cells stained with GFAP:Cy3 are compared with those stained with the isotype control.

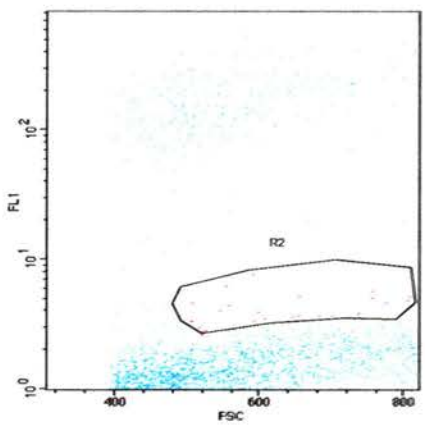
periods, could eliminate this small amount of fluorescence. Due to the unpredictability of availability of the FACS sorter, it is often necessary to store cells after staining and before sorting. For this reason the anti-GFAP antibody conjugated to Cy3 is not suitable. A new anti-GFAP antibody conjugated to AlexaFluor 488 was obtained. This antibody was used to stain a single cell suspension of human brain cells. The results are shown in figure 5.11. Again, a dot plot revealed a new population better than a histogram plot. 40% of anti-GFAP stained cells fall within gate R2 compared to just 1% of isotype control stained cells. This antibody can therefore be used to FACS sort human brain cells.

Microglia are thought to be the main brain cell infected by HIV *in vivo*. It would therefore be very useful to purify microglia from a HIV infected brain and assess levels of infection. An antibody which has previously been used for identification of microglia is CD11b. CD11b (also known as complement receptor 3 or CD18), is a member of the integrin family of adhesion receptors which plays an important role in mediating the transmigration of leukocytes (Ehlers, 2000). In addition, CD11b acts as a pattern recognition receptor for various endogenous and exogenous molecules and mediates cellular activation. An anti CD11b antibody conjugated to FITC was used. Initially the antibody was tested on the cell line J774 which had previously been shown to express CD11b (Zeger et al., 1990). As shown in figure 5.12, approximately 95% of cells stained with the antibody fell within the M1 marker of fluorescence. This is compared to only 1% of cells stained with an isotype control. The antibody was next used to stain a single cell suspension



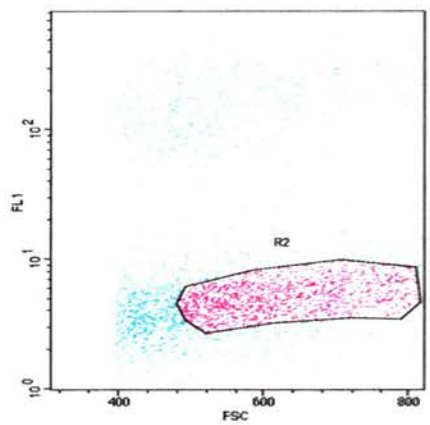
Region	Events	% Total
R1	2770	16.91
R2	27	0.16

Unstained



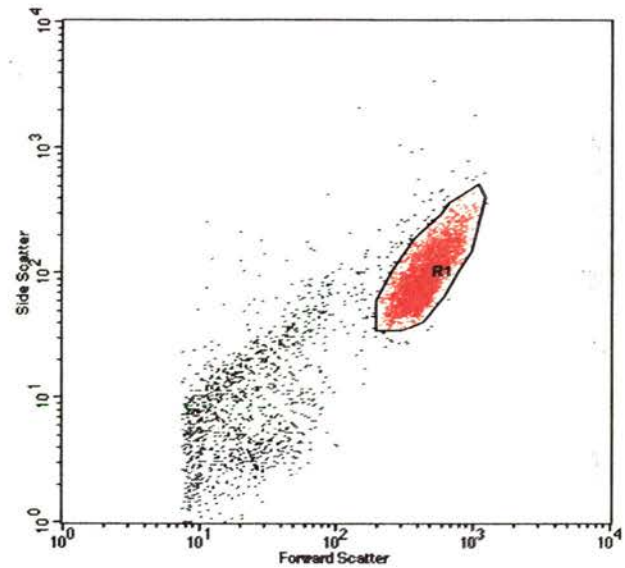
Region	Events	% Gated	% Total
R1	2770	100.00	16.91
R2	26	0.94	0.16

GFAP AlexaFlour488



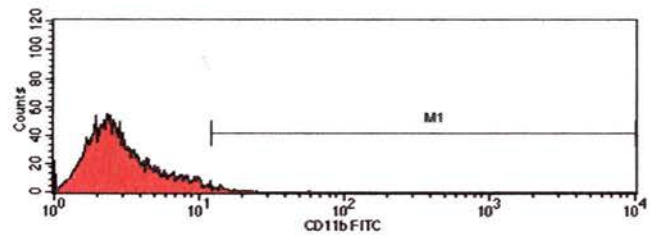
Region	Events	% Gated	% Total
R1	2621	100.00	15.32
R2	1050	40.06	6.14

Figure 5.11: Flow cytometric analysis of human brain cells stained with directly conjugated antibody GFAP:AlexaFlour 488. Gate R1 was defined as previously. Histogram plots comparing events within R1 showed little increase in fluorescence when cells stained with GFAP:AlexaFlour were compared with those stained with the isotype control (data not shown). However, a dot plot of fluorescence against forward scatter, revealed that a new population arises in the GFAP:AlexaFlour stained cells. This population was defined on the dot plot by gate R2. When cells stained with the isotype control are analysed approximately 1% of events fall with this gate. When cells stained with GFAP:AlexaFlour 488 are analysed this percentage rises to approximately 40%.



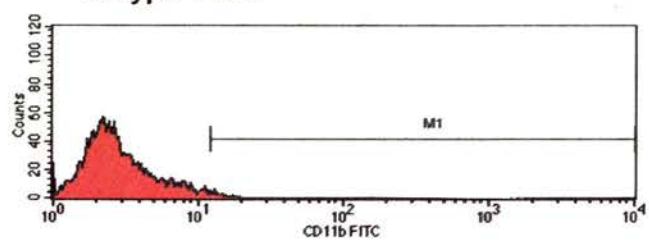
Region	Events	% Total
R1	4891	79.77

Unstained



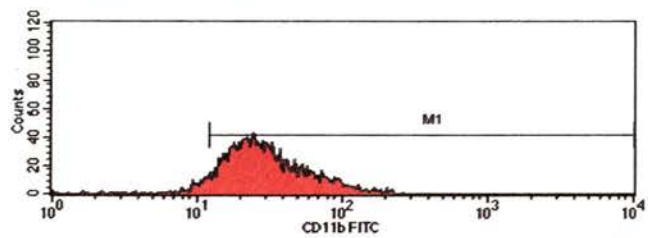
Marker	Events	% Gated	% Total
All	4893	100.00	77.04
M1	71	1.45	1.12

Isotype FITC



Marker	Events	% Gated	% Total
All	4891	100.00	79.77
M1	51	1.04	0.83

CD11b FITC



Marker	Events	% Gated	% Total
All	4856	100.00	75.25
M1	4591	94.54	71.15

Figure 5.12: Flow cytometric analysis of macrophage cell line (J774).Analysis of fluorescence was carried out on events which lay within the R1 gate as defined on the side versus forward scatter dot plot. Events lying out with this gate were presumed to be dead cells or cellular debris. On the histogram plots of unstained cells or cells stained with the isotype control it can be seen that approximately 1% of cells lie within the M1 marker. On the histogram plot of cells stained with CD11b:FITC approximately 95% of events lie within the M1 marker.

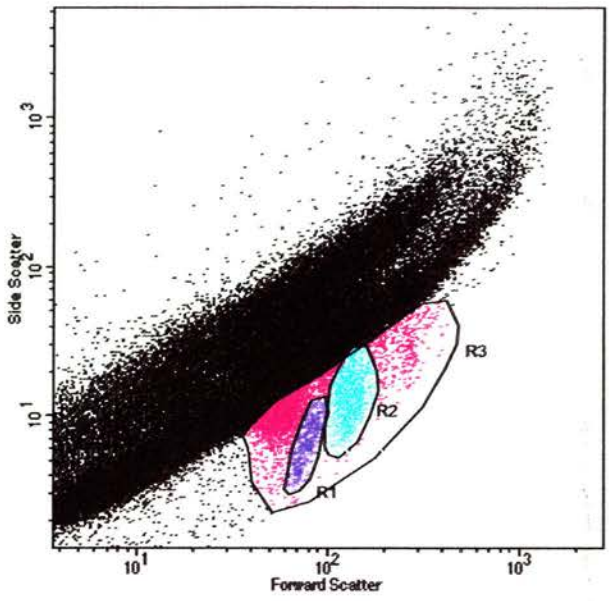
of murine brain cells. As shown in figure 5.13, cells falling within gate 3 show an increased fluorescence when anti-CD11b stained cells are compared with those stained with an isotype control. 30% of anti-CD11b cells fall within the M1 marker compared to only 1% of isotype control stained cells. The antibody was next used to attempt to select human microglia cells. The results of this experiment are shown in figure 5.14. Unfortunately, it appears that this antibody was not able to stain human CD11b effectively and thus could not be used for separation of these cells.

Another antigen which has been used for immunohistochemical identification of human microglia cells is CD68. CD68 is a transmembrane protein found in the endosomal membranes of monocytic cells (Gordon, 1999). It's functions are not fully understood but may include enhancing phagocytic uptake of oxidised low density lipoprotein (Ramprasad et al., 1995). An anti-CD68 antibody conjugated to FITC was obtained. As CD68 is intra-cellular, cells were permeabilised with ethanol prior to staining. This antibody identified a population of cells from a single cell suspension of human brain (figure 5.15). Approximately 4% of anti-CD68 stained cells fall within gate R1 compared to just 0.4% of cells stained with the isotype control. It was therefore decided to use this antibody for subsequent cell sorting.

5.3.6 Analysis of sorted samples

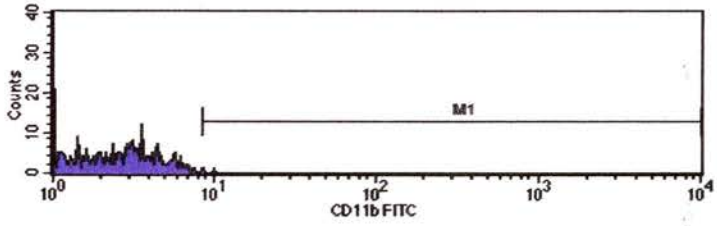
Having optimised the protocol to be used, HIV infected brains were obtained for cell sorting. Clinical details of the study subjects from which tissue was used are given in table 5.2. Cells were stained using either the FITC conjugated anti-CD68 antibody or the AlexaFluor 488 conjugated anti-GFAP

Figure 5.13: Flow cytometric analysis of mouse brain cells. Analysis of fluorescence was carried out on events which lay within the either the R1, R2 or R3 gates as defined on the side versus forward scatter dot plot. These gates were defined as they contain events which showed a significant increase in fluorescence when cells stained with CD11b:FITC are compared to unstained cells or cells stained with the isotype control. Gate R3 includes both the R! and R2 gates as well as some additional events. It was selected in order to investigate if using this larger gate allowed additional CD11b positive cells to be detected. On the histogram plots of unstained cells or cells stained with the isotype comtrol it can be seen that less than 1% of cells lie within the M1 marker for each of the gates. On the histogram plot of cells stained with CD11b:FITC approximately 12%, 62% and 30% of events lie within the M1 marker for cells within gates R1, R2 and R3 respectively. The total number of cells identified as CD11b positive was 74, 869 and 1079 for gates R1, R2 and R3 respectively. Thus, gate R2 contains the greatest percentage purity of CD11b positive cells however using gate R3 allows the greatest total number of CD11b positive cells to be identified.

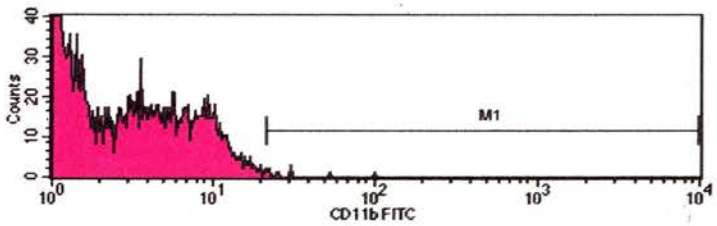


Region	Events	% Total
R1	568	0.57
R2	1599	1.60
R3	3335	3.34

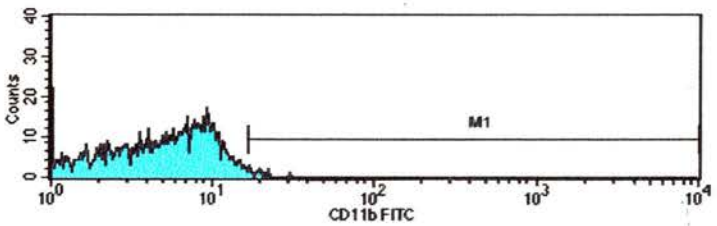
Unstained



Marker	Events	% Gated	% Total
All	647	100.00	0.65
M1	2	0.31	0.00

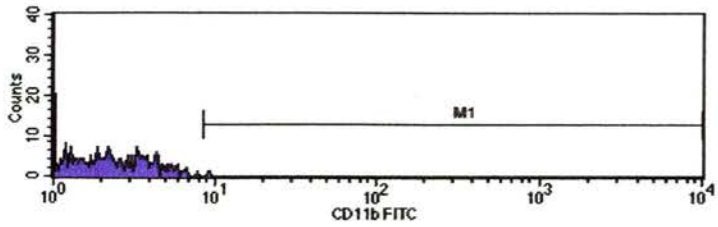


Marker	Events	% Gated	% Total
All	1788	100.00	1.79
M1	13	0.73	0.01

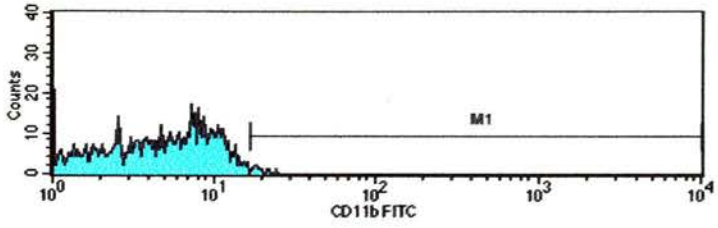


Marker	Events	% Gated	% Total
All	4139	100.00	4.14
M1	27	0.65	0.03

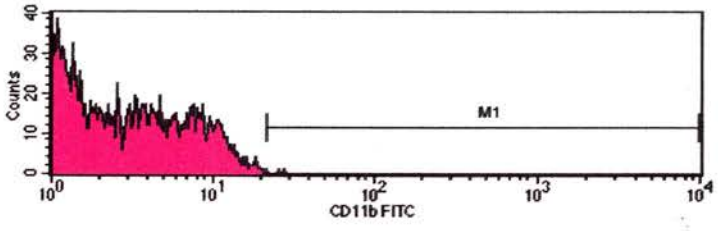
Isotype FITC



Marker	Events	% Gated	% Total
All	568	100.00	0.57
M1	2	0.35	0.00

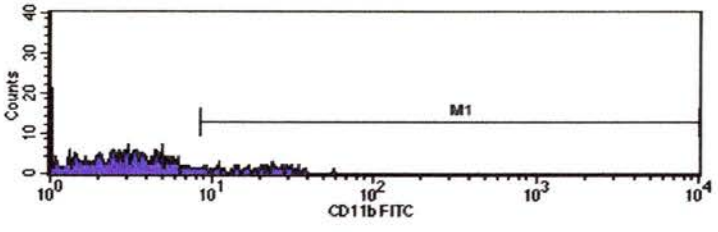


Marker	Events	% Gated	% Total
All	1599	100.00	1.60
M1	15	0.94	0.02

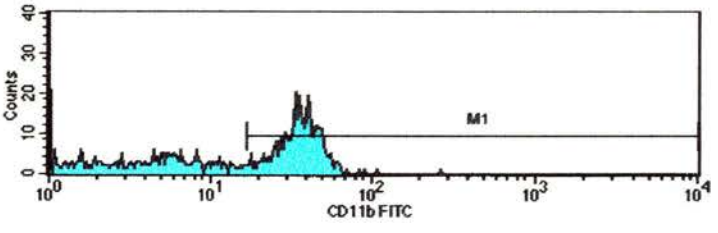


Marker	Events	% Gated	% Total
All	3335	100.00	3.34
M1	7	0.21	0.01

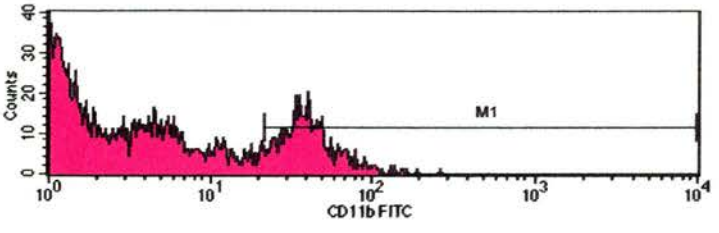
CD11b FITC



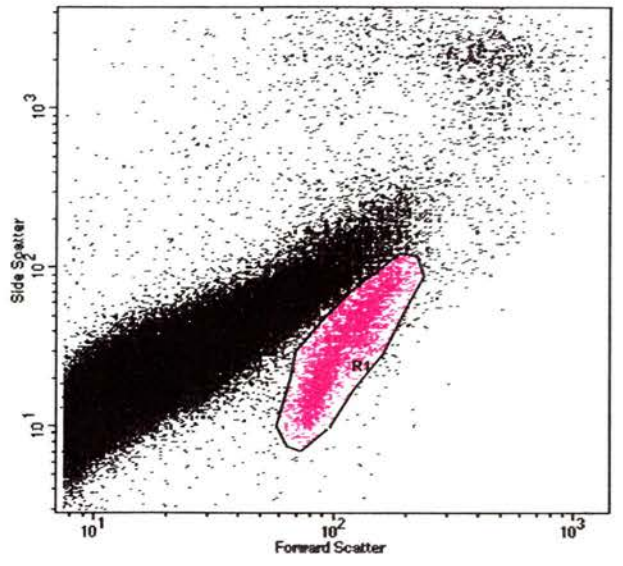
Marker	Events	% Gated	% Total
All	635	100.00	0.64
M1	74	11.65	0.07



Marker	Events	% Gated	% Total
All	1400	100.00	1.40
M1	869	62.07	0.87

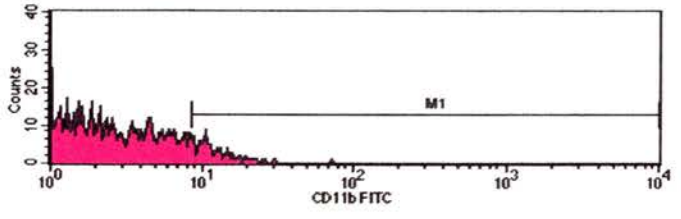


Marker	Events	% Gated	% Total
All	3600	100.00	3.60
M1	1079	29.97	1.08



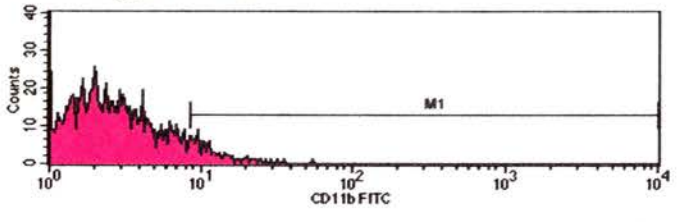
Region	Events	% Total
R1	2933	3.11

Unstained



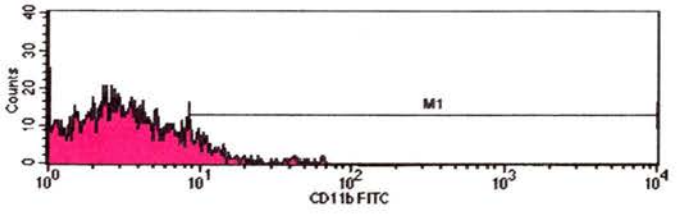
Marker	Events	% Gated	% Total
All	2933	100.00	3.11
M1	32	1.09	0.03

Isotype FITC



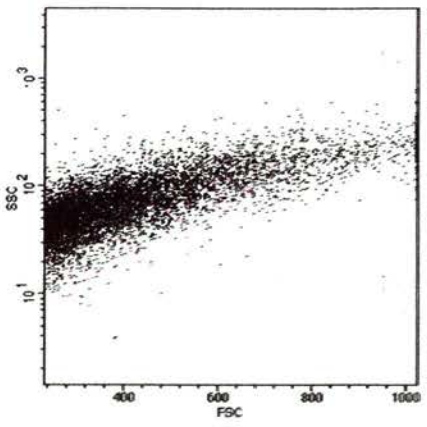
Marker	Events	% Gated	% Total
All	2883	100.00	4.55
M1	28	0.97	0.04

CD11b FITC



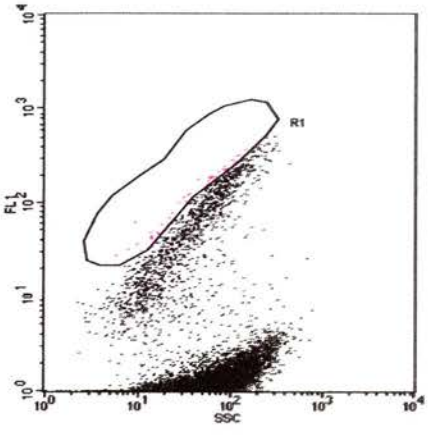
Marker	Events	% Gated	% Total
All	3096	100.00	3.50
M1	240	7.75	0.27

Figure 5.14: Flow cytometric analysis of human brain cells. Analysis of fluorescence was carried out on events which lay within the R1 gate as defined on the side versus forward scatter dot plot. On the histogram plots of unstained cells or cells stained with the isotype control it can be seen that approximately 1% of cells lie within the M1 marker. On the histogram plot of cells stained with CD11b:FITC approximately 7.75% of events lie within the M1 marker.



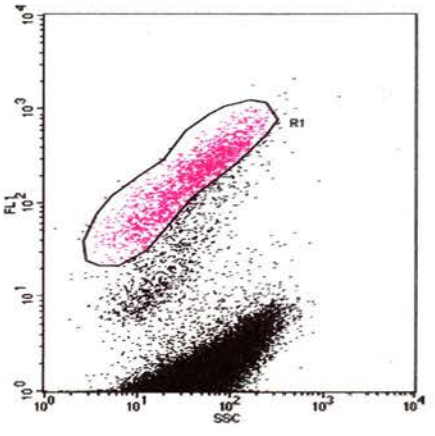
Region	Events	% Total
R1	77	0.39

Isotype FITC



Region	Events	% Gated	% Total
R1	77	0.39	0.39

CD68 FITC



Region	Events	% Gated	% Total
R1	1745	4.43	4.43

Figure 5.15: Flow cytometric analysis of human cells. No gate was on the side versus forward scatter plot in order to allow the maximum number of positive cells to be identified. A histogram plots of cells stained with CD68:FITC did not show significantly higher fluorescence compared to unstained cells or cells stained with the isotype comtrol (data not shown). However, a dot plot of fluorescence against side scatter reveals a new population of events (gate R1) in cells stained with CD68:FITC compared with those stained with isotype control. When cells stained with CD68:FITC are examined, 4.43% of cells fall within gate R1 compared to only 0.39% when cells stained with the isotype control are used.

Study subject	Age	Sex	Cause of death	Risk group	No. of lymphocytes per µl blood		Time before death of last lymphocyte count
					CD4	CD8	
150	32	M	AIDS	MSM	nk	nk	nk
207	43	M	OD	IVDU	335	649	1m18d

Table 5.2: Clinical background of study subjects. OD: Over-dose; MSM: Men who have sex with men; IVDU: Intra-venous drug user; nk: not known

Study subject	Tissue/Cell	Purity	Proviral load
150	Brain	n/a	7.2 per 1 x 10^6 cells
	Spleen		9540 per 1 x 10^6 cells
	Astrocytes	98%	<1 per 1 x 10^4 cells
	Microglia	99%	<1 per 1 x 10^5 cells
207	Brain	n/a	0.16 per 1 x 10^6 cells
	Spleen		5250 per 1 x 10^6 cells
	Astrocytes	98%	<1 per 3 x 10^5 cells
	Microglia	93%	<1 per 1 x 10^4 cells

Table 5.3: Calculated proviral loads and cell purity. As no provirus was detected in any of the sorted cells the proviral load must be less than 1 copy per the number of cells tested. The percentage purity values indicate the proportion of cells positive for the relevant antigen (GFAP for astrocytes and CD68 for microglia) during FACS.

antibody as these antibodies had given the optimum staining as described above. Both antibodies have almost identical absorption and emission spectra (figure 5.16) and thus single staining was necessary. 10% of cells were left unstained, 10% were stained with the isotype control and of the remainder of the cells, 50% were stained with the anti-CD68 antibody and 50% were stained with the anti-GFAP antibody. The population of astrocytes to be collected was defined on fluorescence against forward scatter dot plot. The population of microglia to be collected was defined on a fluorescence against side scatter dot plot. These plots enabled population with maximum purities to be collected. A suspension of single cells was obtained as shown in figure 5.17. The proportion of contaminating events of the population obtained was calculated by dividing the proportion of isotype control stained cells which fell within the defined gate by the proportion of cells stained with the antibody of interest which fell within the defined gate. This data was used to calculate the purity of the populations as shown in table 5.3.

Purified cells were pelleted and their DNA extracted. The DNA concentration was assessed using a spectrophotometer. Nested β-actin PCRs were positive at dilutions of DNA which represented an average of one cell per PCR tube (data not shown). However, despite carrying out many hundreds of reactions, no nested V3 PCRs were positive.

As the brains used were derived from study subjects who had died without AIDS defining illnesses, it was possible that any HIV present in the brain was simply due to contaminating blood and that a separate brain cell tropic variant had not emerged. To test this hypothesis DNA was extracted from

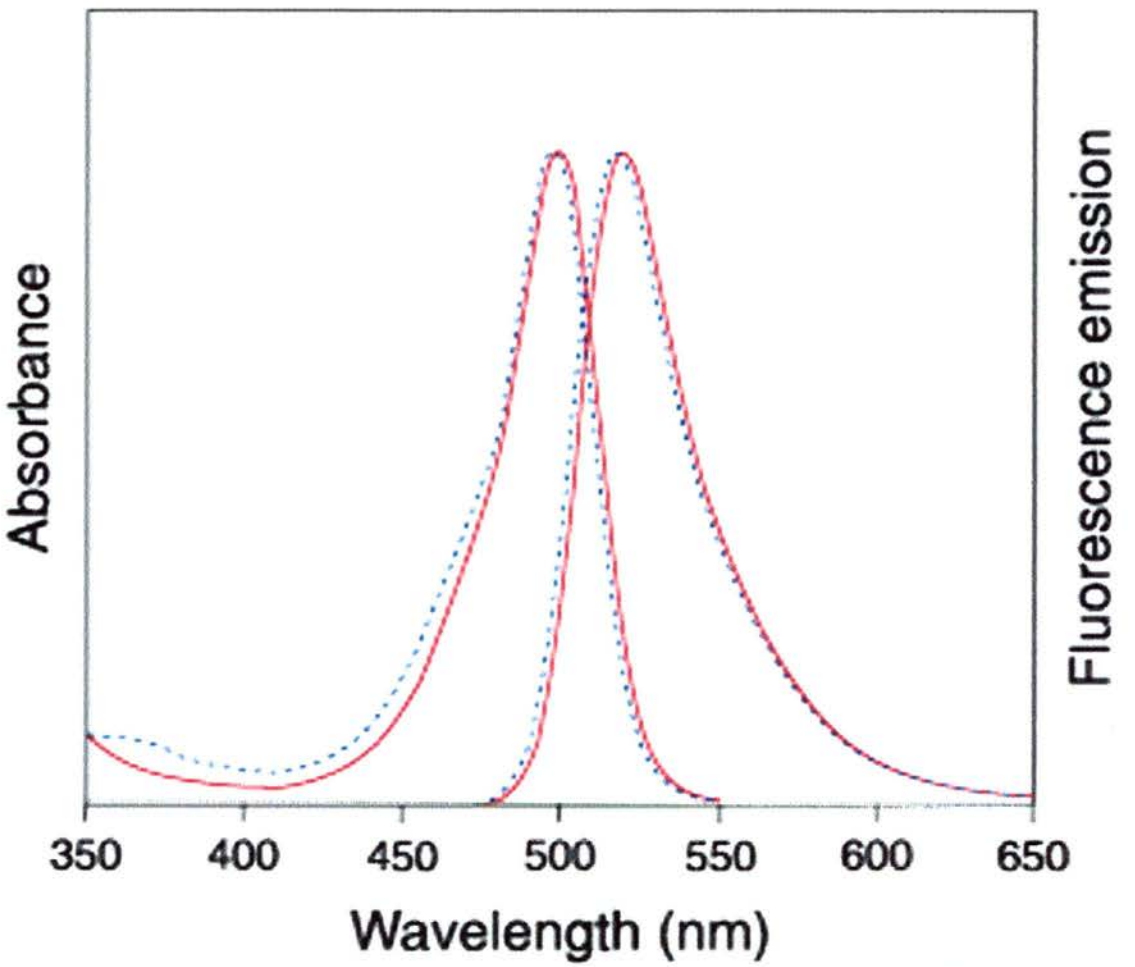


Figure 5.16: Absorption and emission spectra of Alexa Fluor 488 (dashed line) and FITC (continuous line) (Haughland, 1999). Please note that the intensity of fluorescence has not been shown to scale in order to allow comparison of spectral characteristics. Alexa Fluor 488 emits a higher intensity of fluorescence than FITC.

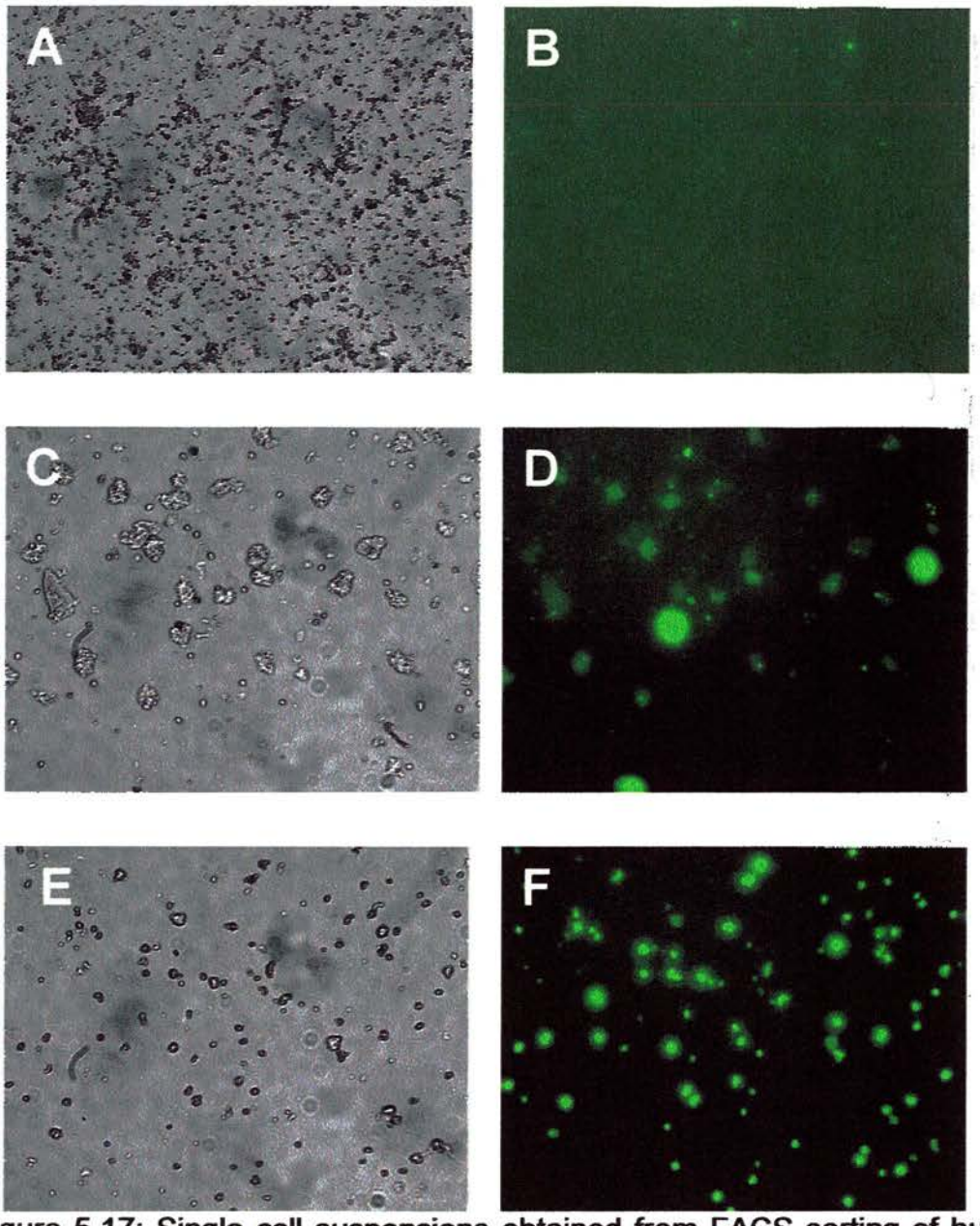


Figure 5.17: Single cell suspensions obtained from FACS sorting of human brain cells visualised at 20X magnification using normal (A, C and E) or UV (B, D and F) illumination. A and B: Cell suspension used for FACS sorting; C and D: GFAP positive cells stained with anti-GFAP antibody conjugated to AlexaFluor 488 ; E and F: CD68 positive cells stained with anti-CD68 antibody conjugated to FITC. Note the large size and irregular morphology of the GFAP positive cells. This is as expected for astrocytes.

portions of frozen brain and spleen samples from the same study subjects. Limiting dilution V3 PCR was carried out and V3 products amplified from single templates were sequenced. Proviral loads were calculated for each tissue and the sequences were compared phylogenetically.

As shown in table 5.3, the proviral loads in the brains of both study subjects were extremely low. In addition the phylogenetic tree obtained using sequences from study subject 150 shows that the sequences obtained from brain and spleen are completely interspersed (figure 5.18). This supports the hypothesis that the sequences amplified from the brain were derived from blood contaminants. Despite carrying out many hundreds of PCRs, only one sequence was obtained from the brain of study subject 207. As shown in figure 5.19, this sequence appeared "spleen-like" when compared phylogenetically with sequences obtained from the spleen. These results suggest that there was almost no HIV provirus in the brain of this individual and that any virus present was likely to be contained in resident blood vessels.

5.4 Discussion

In this study a method was devised to isolate pure populations of astrocytes and microglia from fresh human brain material obtained at post mortem. This procedure could be adapted to also purify other cell types including oligodendrocytes and neurons. The procedure involved first homogenising the tissue to produce a single cell suspension. Broken cell processes and cellular debris were removed using a two step Percoll gradient. The purified brain cells could then be used for cell staining using antibodies specific for

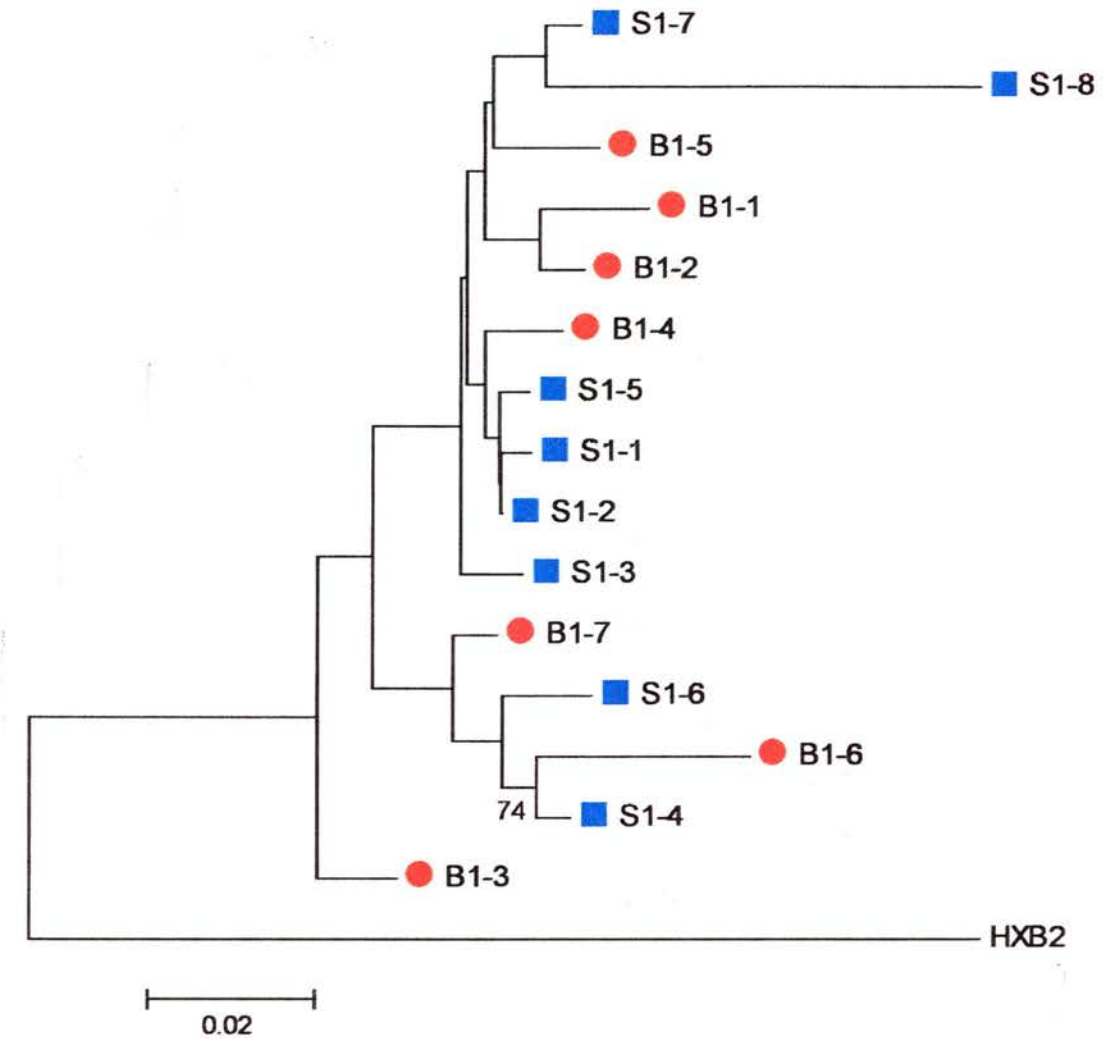


Figure 5.18: Phylogenetic tree showing the genetic relationships between V3 sequences obtained from brain and spleen tissue from study subject 150. Spleen sequences are represented by blue squares while brain sequences are represented by red circles.

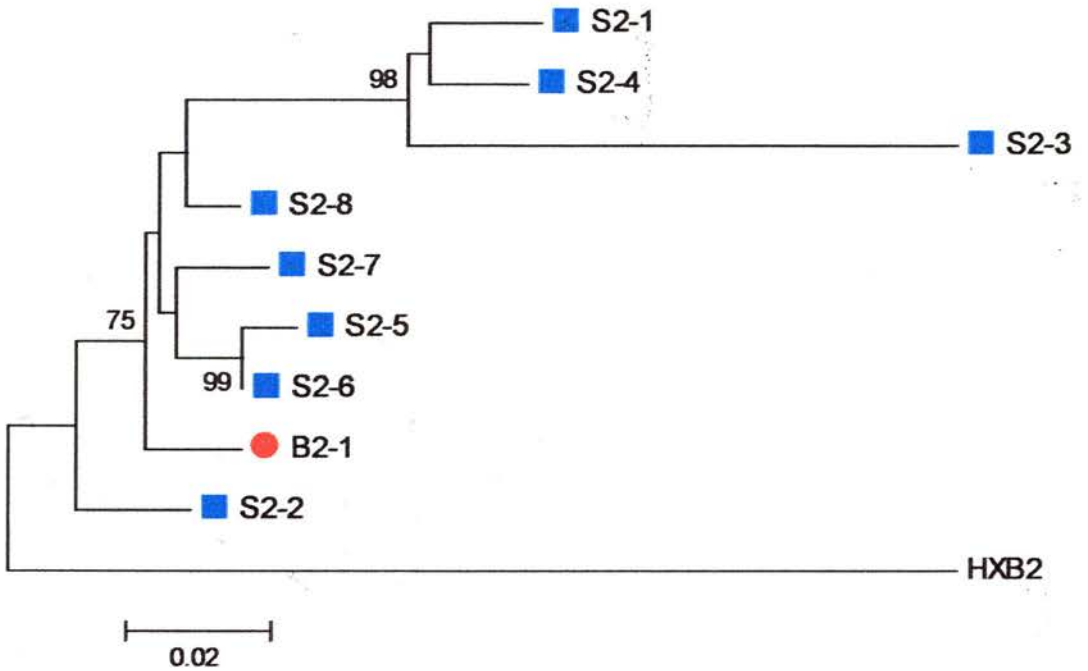


Figure 5.19: Phylogenetic tree showing the genetic relationships between V3 sequences obtained from brain and spleen tissue from study subject 207. Spleen sequences are represented by blue squares while brain sequences are represented by red circles.

cell surface markers. A number of antibodies were tested for their ability to stain brain cells. Testing was carried out first on cell lines, then on murine brain cells and finally using post mortem human brain material. A gentle method of ethanol fixation was used. This allowed the staining of intra-cellular antigens while maintaining the integrity of cellular DNA. The use of primary antibodies followed by fluorochrome conjugated secondary antibodies resulted in an high level of non-specific staining. For this reason directly conjugated antibodies were chosen. The antibodies which were best able to select microglia and astrocytes were anti-CD68 and anti-GFAP respectively.

Cells from HIV positive brain samples were separated and the extracted DNA tested for HIV sequences using PCR. None of the DNA from the separated cells was positive for HIV proviral DNA. Limiting dilution PCR carried out on DNA extracted from whole brain tissue revealed that the material used had a very low proviral load and that sequences found in the brain grouped phylogenetically with those found in the spleen. Taken together, these results suggest that in the study subjects investigated, there was no separately replicating population of HIV in the brain. It is likely that the presence of V3 template within the brain tissue was due to contamination of the tissue with blood.

It would be very interesting to carry out this work on brain material from a study subject who had died of AIDS particularly where there was pathological evidence of HIVE. However, obtaining such material in current times is extremely difficult. For example, in Edinburgh there has only been one post

mortem on a subject with HIVE, where consent has been obtained for research on organs, since the advent of treatment with HAART in 1996 (Bell, J personal communication).

One possible option would be to carry out similar studies using an animal model of HIVE. A number of lentiviral infections have been studied as potential models of HIVE (reviewed in Burudi and Fox, 2001). These include feline immunodeficiency virus (FIV) infection of cats, Visna-Maedi virus infection of sheep and caprine arthritis encephalitis virus infection of goats. Strains of SIV are found in various African monkey species and do not appear to cause pathology in their natural host. However, infecting a previously unexposed host species with SIV can result in an immunodeficiency disease similar to AIDS. For example, the model which has been used most frequently is infection of macaques with a sooty mangabey strain of SIV. Different strains of SIV differ in their ability to cause neurological disease in macaques. Passage of virus through microglial cell lines along with the use of recombinant DNA technology has allowed neurotropic variants to be produced. In one particularly effective model of neurovirulence a neurotropic clone, SIV_{MAC}17E-Fr, is inoculated along with a potent immunosuppressive virus, SIV_{MAC}B670/Delta, into pig-tailed macaques (Mankowski et al., 2002). This results in a consistent accelerated model of HIVE with over 90% of animals developing encephalitis within 6 months of infection. Such models produce a disease which closely mirrors HIVE in many ways. However, there are some differences in pathology. In particular there have been no reports of infection of cell types other than

microglia/monocytes in macaque models. Thus using this model to investigate the extent of infection of different brain cells could be misleading if previous reports of infection of astrocytes and neuron in humans are correct. To circumvent this problem, some studies have used SIV/HIV (SHIV) chimeras to infect macaques (Joag et al., 1997; Liu et al., 1999). It is thought that this might lead to a disease which more closely mimics that found in humans.

If infected brain material was obtained either from macaque or human study subjects, a number of aspects of neuropathogenesis could be investigated. As attempted in this study, HIV proviral loads could be determined for different cell types. This would help solve the question of which cell types are infected and to what extent. In addition, *env* genes could be amplified from different cell types and used to produce chimeric viruses as described in chapter 3. These could be investigated to identify the specific factors (both viral and host) which determine cell tropism.

Another possibility would be to extract RNA from populations of sorted cells. This would require the tissue to be processed and fixed soon after death of the subject in order to prevent RNA degradation. Thus, logistically, this may only be possible when using an animal model. Extracted RNA could be assessed using a microarray to investigate regulation of specific genes. For example, it would be informative to monitor levels of mRNA for peptides involved in regulating oxidative stress; a process thought to be implicated in HIVE associated pathology (reviewed in Mollace et al, 2001; Kaul et al, 2001). Expression of genes such as nitric oxide synthase and superoxide dismutase

(which acts to reduce superoxide anion) could be assessed in different brain cells. The two compounds which are regulated by these enzymes, nitric oxide and superoxide anion, react together to form the highly damaging product peroxynitrite which may contribute to the development of HIVE. The release of these and other oxidative compounds is promoted by the proinflammatory cytokines such as IL-1β and TNFα. Also, certain chemokines such as MCP-1 may play a role in recruiting monocytes/microglia to areas of inflammation. Assessing the mRNA levels for these and other immune molecules in specific brain cell types could dramatically further our understanding of the pathogenesis of HIVE.

Two recent studies have used laser capture microdissection to capture cells from HIVE brain material and assessed them for HIV infection (Torres-Munoz et al., 2001; Trillo-Pazos et al., 2003). Both studies report infection of neurons while the more recent paper also describes frequent infection of microglia and astrocytes. Further studies using this exciting new technique will be required to confirm these findings. As techniques capable of investigating at such a "micro" level are developed, new details about the cellular pathogenesis of HIV should emerge. Hopefully, within a few years the controversy of which cells are infected and to what extent will be solved. In addition, and perhaps more importantly, far more detailed investigations of the processes which lead to neuronal damage may be possible.

Chapter 6: Conclusions

Despite an unprecedented amount of research, there are still many aspects of HIV pathogenesis which remain incompletely characterised. One such area is cellular tropism. In its broadest sense, this topic encompasses a variety of research foci including characterisation of cells infected *in vivo*, characterisation of the genotypic/phenotypic nature of variants infecting different cell types/ anatomical sanctuaries and investigation of the host and viral factors which determine differential tropism. In this project a variety of techniques were employed in an attempt to further address these issues.

The aim of the first part of the project was to produce an *in vitro* system which would allow more accurate characterisation of viral and host factors involved in cellular tropism. HIV requires a co-receptor in order to infect a cell (Feng et al., 1996). *In vivo*, the main co-receptors used are CCR5 and CXCR4. Variants can thus be defined as CCR5 using (R5), CXCR4 using X4 or dual tropic (X4R5). These differences account for some of the differences in cellular tropism between viral variants however, it appears that other factors may also play a role (Yi et al., 1998). In order to fully address the factors determining cellular tropism, various *in vitro* approaches have been used. The principle of all these methods is to amplify the virus and then assess its ability to infect different cell types. The simplest method of viral amplification is to culture the virus directly from infected blood/tissue. The problem with this method is that culturing *in vitro* exerts a selective pressure on the population. Due to HIV's inherently rapid rate of evolution and the presence of a heterogenous starting population, the virus obtained may not accurately represent the virus present *in vivo*. In addition, it is difficult to

correlate differences in tropism to genetic factors when a mixed population of virus is used. Methods which employ amplification of viral sequences in prokaryotic cells have been used. The *env* gene, (the major viral determinant of tropism) can be amplified either alone and used to produce pseudotyped virions or the gene can be inserted into and amplified in the context of the entire HIV backbone which can then be used to produce infectious virus. Such methods have the advantage that the population of virus produced will be homogenous and differential growth of variants should not influence the results. However, amplification of viral sequences in prokaryotic cells can also exert a selective pressure of the sequence. In particular various HIV genes have been demonstrated to be toxic to *E. coli* (Kusumi et al., 1992; Baum et al., 1990). This phenomenon can lead to preferential growth of bacteria containing defective genomes (Peden, 1992). Thus a method for production of viruses which does not involve amplification of *env* in prokaryotic cells would be valuable. A novel vector was produced which allowed the production of chimeric viruses containing exogenous *env* genes and the EGFP gene. Three methods using this vector to produce infectious virus without amplification of env in prokaryotic cells were investigated. All methods were able to generate infectious virus. The quantity of virus produced was low and thus further optimisation will be required in order to generate sufficient virus for effective studies of viral tropism.

The second part of this project sought to further characterise the drug resistance genotype of variants infecting two compartments- the brain and the lymphoid system. Anti-retroviral drugs have poor penetration into the

CNS. For this reason, there has been much discussion within the scientific community regarding the distribution of drug resistance mutations within the brain. Two alternative hypotheses have been proposed. One theory is that poor penetration may lead to sub-optimal drug levels which could hasten the evolution of drug-resistant variants in the brain. Alternatively, drugs may be retarded from entry to such a degree that only negligible amounts will be present in the brain. This could lead to the brain becoming a sanctuary site for drug-sensitive variants which could not survive in the lymphoid system. To address this issue post-mortem brain and lymphoid tissue was obtained from four HIV positive individuals. A fragment of RT was amplified by PCR from the proviral templates present in the tissue. Approximately eight limiting dilution fragments were amplified and sequenced for each tissue. Examination of the aligned sequences revealed that in three out of four subjects there were no differences in the pattern of drug resistance mutations between the two compartments. However, in one subject there were significantly more drug resistance mutations in the lymphoid-derived sequences than in those amplified from the brain. These results are consistent with two previous studies which found either no difference in distribution of drug resistance mutations between brain and lymphoid derived sequences or an overrepresentation of drug resistance muations in lymphoid derived sequences (Wong et al., 1997); (McClernon et al., 2001). A portion of the PR gene was amplified and sequenced from the tissue of two subjects who had not taken PIs. These results will be used as a control for a future study investigating the distribution of PI resistance mutations in tissues of

subjects who have taken PIs.

The final part of this project sought to characterise the cellular location of HIV in the brain. Conflicting results have emerged regarding which CNS cells are infected with HIV. It is generally accepted that the major cell types infected by HIV within the brain are microglia and macrophages (Vazeux et al., 1987; Koenig et al., 1986). However various investigators have reported infection of astrocytes or even neurons (Saito et al., 1994; Bagasra et al., 1996; Trillo-Pazos et al., 2003). Methods which could be used for the bulk isolation of highly pure cell populations from post-mortem HIV positive brains were investigated. An important consideration was the preservation of cellular DNA so that subsequent PCRs could be carried out. Thus the separated cell populations can be tested for the presence of HIV provirus. A number of techniques for cell separation were attempted. Optimal results were obtained by a process involving removal of cellular debris by density centrifugation; permeabilisation using ethanol; staining for intra-cellular antigens and finally FACS sorting. This method was used to obtain highly pure populations of microglia and astrocytes from post-mortem brains. Extracted DNA from these populations was negative by PCR for HIV provirus. It is assumed that this result was due to the low proviral loads in the brain material used.

Appendix I: Log of Transfection experiments

Start date	Genetic material transfected	Fluorescence in 293T cells	Fluorescence in susceptible cells	Outcome/ Comments
16/07/02*	1. NL4.3(NL4.3*env*)EGFP (C)	✔	✔	Syncytia apparent in C8166 cells after 3 days
	2. pNL4.3(NL4.3*env*)EGFP (M1)	x	x	
	3. pNL4.3(Δ*env*)EGFP (C)	x	x	
	4. pEGFP (C)	✔	x	
18/07/02*	1. pNL4.3(NL4.3*env*)EGFP (C)	✔	✔	Fluorescence in 293T cells transfected with ligation mix far more faint and sparse but apparent after 3 days
	2. pNL4.3(NL4.3*env*)EGFP (M1)	✔	✔	
21/07/02	1. pNL4.3(NL4.3*env*)EGFP (C)	✔	✔	Increased quantity of ligated DNA used- little increase in quantity of infectious virus
	2. pNL4.3(NL4.3*env*)EGFP (M1)	✔	✔	
10/08/02	1. pNL4.3(NL4.3*env*)EGFP (C)	✔	✔	Use of Sf162, YU2 and 89.6 *env* genes allowed expression of EGFP in transfected 293T cells but didn't appear capable of infectious virus production- perhaps non-functional copies of gene?
	2. pNL4.3(NL4.3*env*)EGFP (M1)	✔	✔	
	3. pNL4.3(Sf162*env*)EGFP (M1)	✔	x	
	4. pNL4.3(YU2*env*)EGFP (M1)	✔	x	
	5. pNL4.3(HXB2*env*)EGFP (M1)	✔	✔	
	6. pNL4.3(89.6*env*)EGFP (M1)	✔	x	
5/10/02	1. pNL4.3(YU2*env*)EGFP (C)	✔	✔	293T cells transfected with ligation mix appear dead-
	2. pNL4.3(YU2*env*)EGFP (M1)	x	x	

				perhaps due to toxicity of DNA
30/10/02	1. pNL4.3(YU2*env*)EGFP (C)	x	x	Later determined that YU2 clone used as PCR template for this experiment contained defective *env* gene
	2. pNL4.3(YU2*env*)EGFP (M1)	x	x	
14/11/02	1. pNL4.3aEGFP (C)	✔	✔	After 1 week most cells appear infected by the HXB2 *env* containing virus. Virus containing YU2 *env* appears far less infectious. This is as expected for a R5 (slow) variant.
	2. pNL4.3(YU2*env*)EGFP (C)	✔	✔	
	3. pNL4.3(HXB2*env*)EGFP (C)	✔	✔	
18/01/03	1. pNL4.3(YU2*env*)EGFPa (C)	✔	✔	After 1 week, infected PM1 cells apparent but most cells remain uninfected. Virus has limited infectivity.
	2. pNL4.3(YU2*env*)EGFPb (C)	✔	✔	
3/02/03	1. pNL4.3(ADA*env*)EGFPa (C)	✔	✔	S/N from (1) used to produce bulk culture-large amount of infectious virus produced and stored ADA *env* containing virus has "slow" phenotype when compared to viruses with X4 *env* genes (i.e. NL4.3 or HXB2) however it
	2. pNL4.3(ADA*env*)EGFPb (C)	✔	x	
	3. pNL4.3(ADA*env*)EGFPc (C)	✔	✔	
	4. pNL4.3(ADA*env*)EGFPd (C)	✔	x	
	5. pNL4.3(ADA*env*)EGFPe (C)	✔	x	
	6. pNL4.3(RO1*env*)EGFP (C)	✔	x	
	7. pNL4.3(RO2*env*)EGFP (C)	✔	✔	

	8. pNL4.3(RO3*env*)EGFP (C)	✔		✔	appears more infectious than the YU2 *env* containing viruses produced in previous experiments. Some of the study subject-derived *env* gene containing viruses were infectious in PM1 cells.
	9. pNL4.3(LT1*env*)EGFP (C)	✔		x	
	10. pNL4.3(LT2*env*)EGFP (C)	✔		x	
	11. pNL4.3(LT3*env*)EGFP (C)	✔		✔	
12/02/03	1. pNL4.3(ADA*env*)EGFP (M1)	x		x	
19/02/03	1. pNL4.3(RO4*env*)EGFP (C)		N/D		All cells died[#] Presumed to be due to bacterial infection of cells. Later determined to be due to faulty CO_2 regulator on incubator.
	2. pNL4.3(RO5*env*)EGFP (C)				
	3. pNL4.3(RO6*env*)EGFP (C)				
	4. pNL4.3(RO7*env*)EGFP (C)				
	5. pNL4.3(RO8*env*)EGFP (C)				
	6. pNL4.3(RO9*env*)EGFP (C)				
	7. pNL4.3(RO10*env*)EGFP (C)				
	8. pNL4.3(LT4*env*)EGFP (C)				
	9. pNL4.3(LT5*env*)EGFP (C)				
	10. pNL4.3(LT6*env*)EGFP (C)				
	11. pNL4.3(LT7*env*)EGFP (C)				
	12. pNL4.3(LT8*env*)EGFP (C)				
	13. pNL4.3(LT9*env*)EGFP (C)				
	14. pNL4.3(LT10*env*)EGFP (C)				
6/03/03	1. pNL4.3(RO1*env*)EGFP (C)	✔		N/D	All cells died as

		above[#]
2. pNL4.3(RO2*env*)EGFP (C)	✔	
3. pNL4.3(RO3*env*)EGFP (C)	✔	
4. pNL4.3(RO4*env*)EGFP (C)	x	
5. pNL4.3(RO5*env*)EGFP (C)	✔	
6. pNL4.3(RO6*env*)EGFP (C)	✔	
7. pNL4.3(RO70*env*)EGFP (C)	✔	
8. pNL4.3(RO8*env*)EGFP (C)	✔	
9. pNL4.3(RO9*env*)EGFP (C)	✔	
10. pNL4.3(RO10*env*)EGFP (C)	✔	
11. pNL4.3(LT1*env*)EGFP (C)	x	
12. pNL4.3(LT2*env*)EGFP (C)	✔	
13. pNL4.3(LT3*env*)EGFP (C)	✔	
14. pNL4.3(LT40*env*)EGFP (C)	✔	
15. pNL4.3(LT5*env*)EGFP (C)	x	
16. pNL4.3(LT6*env*)EGFP (C)	✔	
17. pNL4.3(LT7*env*)EGFP (C)	✔	
18. pNL4.3(LT8*env*)EGFP (C)	✔	
19. pNL4.3(LT9*env*)EGFP (C)	✔	
20. pNL4.3(LT10*env*)EGFP (C)	✔	
21. pNL4.3(HXB2*env*)EGFP (C)	✔	
22. pNL4.3(ADA*env*)EGFP (C)	✔	
23. pNL4.3(HXB2*env*)EGFP (M2)	✔	

	24. pNL4.3(ADA*env*)EGFP (M2)	✔		
10/03/03	1. pNL4.3(HXB2*env*)EGFP (C)	✔	✔	Far less virus from 293T cells transfected with OE compared with cloned DNA
	2. pNL4.3(ADA*env*)EGFP (C)	✔	✔	
	3. pNL4.3(HXB2*env*)EGFP (M2)	✔	✔	
	4. pNL4.3(ADA*env*)EGFP (M2)	✔	✔	
17/03/03	1. pNL4.3(RO1*env*)EGFP (C)	✔	✔	Similar quantity of infectious virus produced from 293T cells transfected with OE or mixed fragments
	2. pNL4.3(RO2*env*)EGFP (C)	✔	✔	
	3. pNL4.3(RO3*env*)EGFP (C)	✔	✔	
	4. pNL4.3(RO4*env*)EGFP (C)	x	x	
	5. pNL4.3(RO5*env*)EGFP (C)	✔	x	
	6. pNL4.3(RO6*env*)EGFP (C)	✔	✔	
	7. pNL4.3(RO70*env*)EGFP (C)	✔	✔	
	8. pNL4.3(RO8*env*)EGFP (C)	✔	x	
	9. pNL4.3(RO9*env*)EGFP (C)	✔	x	
	10. pNL4.3(RO10*env*)EGFP (C)	✔	x	
	11. pNL4.3(LT1*env*)EGFP (C)	x	x	
	12. pNL4.3(LT2*env*)EGFP (C)	✔	x	
	13. pNL4.3(LT3*env*)EGFP (C)	✔	✔	
	14. pNL4.3(LT40*env*)EGFP (C)	✔	x	
	15. pNL4.3(LT5*env*)EGFP (C)	x	x	
	16. pNL4.3(LT6*env*)EGFP (C)	✔	✔	
	17. pNL4.3(LT7*env*)EGFP (C)	✔	✔	

	18. pNL4.3(LT8*env*)EGFP (C)	x		x	
	19. pNL4.3(LT9*env*)EGFP (C)	x		x	
	20. pNL4.3(LT10*env*)EGFP (C)	✔		x	
	21. frag S/307 (ADA *env*)	x		x	
	22. frag AS/306 (ADA *env*)	x		x	
	23. pNL4.3(ADA*env*)EGFP (M2)	✔		✔	
	24. pNL4.3(ADA*env*)EGFP (M2)	✔		✔	
25/03/03	1. pYFP	✔		N/D	
	2. pNL4.3(HXB2*env*)YFP (M2)	x			
	3. pNL4.3(ADA*env*)YFP (M2)	x			
1/04/03	1. pNL4.3(HXB2*env*)EGFP (M3)	✔		✔	Similar amount of virus produced as using L or OE
	2. pNL4.3(ADA*env*)EGFP (M3)	✔		✔	
17/04/03	1. pNL4.3(P1*env*)EGFP (M3)		N/D		All cells died as previous[#]
	2. pNL4.3(P2*env*)EGFP (M3)				
	3. pNL4.3(P3*env*)EGFP (M3)				
	4. pNL4.3(P4*env*)EGFP (M3)				
	5. pNL4.3(ADA*env*)EGFP (M3)				
23/04/03	1. pNL4.3(P1*env*)EGFP (M3)		N/D		All cells died as previous[#]
	2. pNL4.3(P2*env*)EGFP (M3)				
	3. pNL4.3(P3*env*)EGFP (M3)				
	4. pNL4.3(P4*env*)EGFP (M3)				
	5. pNL4.3(ADA*env*)EGFP (M3)				

29/04/03	1. pNL4.3(HXB2*env*)YFPa (C)	x	N/D	No fluorescence seen
	2. pNL4.3(HXB2*env*)YFPb (C)	x		
	3. pNL4.3(ADA*env*)YFPa (C)	x		
	4. pNL4.3(ADA*env*)YFPb (C)	x		
NEW INCUBATOR PURCHASED				
20/05/03	1. 1.2 µg pNL4.3(Δ*env*)EGFP linearised	x	N/D	Experiment carried out to optimise quantity of DNA used
	2. 0.3 µg pNL4.3(Δ*env*)EGFP linearised	x		
	3. 1.2 µg *env*EGFP fragment	x		Determined that high concentration (1.2 µg per well) of cut vector causes cell death
	4. 0.3 µg ADA*env*EGFP fragment	x		
	5. 1.2 µg pNL4.3(Δ*env*)EGFP linearised + 1.2 µg *env*EGFP fragment	✔		
	6. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.3 µg *env*EGFP fragment	✔		
	7. 0.4 µg NL4.3(ADA*env*)EGFP	✔		
	8. 0.1 µg pNL4.3(ADA*env*)EGFP	✔		
	9. Transfast reagent only	x		
	10. Media only	x		
8/06/03	1. 0.3 µg pNL4.3(Δ*env*)EGFP linearised	x	x	Experiment carried out to optimise quantity of *env*EGFP DNA used
	2. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg ADA*env*EGFP fragment	✔	✔	
	3. 0.3 µg pNL4.3(Δ*env*)EGFP linearised- + 1.5 µg ADA*env*EGFP fragment	✔	✔	Determined that high concentration (1.5 µg per well) yielded less virus than lower concentration
	4. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg HXB2*env*EGFP	✔	✔	

	fragment			(0.9 µg)
	5. 0.3 µg pNL4.3(Δ*env*)EGFP linearised- + 1.5 µg HXB2*env*EGFP fragment	✔	✔	
11/06/03†	1. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg P1*env*EGFP fragment	✔	✔	This determined that the rec method could be used to produce viruses with various *env* genes- i.e. patient derived and non-B subtypes
	2. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg P2*env*EGFP fragment	✔	✔	
	3. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg P3*env*EGFP fragment	✔	✔	
	4. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg subtypeA*env*EGFP fragment	✔	✔	
	5. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg subtypeC*env*EGFP fragment	✔	✔	
	6. 0.3 µg pNL4.3(Δ*env*)EGFP linearised + 0.9 µg HXB2*env*EGFP fragment	✔	✔	

Abbreviations: (C): Cloned plasmid DNA; (M1): Non-cloning method 1 (Ligation mixture); (M2): Non-cloning method 2 (Overlap extension product); (M3): Non-cloning method 3 (mixture of the products needed to produce virus by the recombination method- i.e. linearised vector and an *env*EGFP fragment); S/N: Supernatant from transfected 293T cells; N/D: Not done; RO1-10 and LT1-10 are limiting dilution *env* products produced using template DNA from the right occipital and left temporal brain lobes of a single study subject respectively.

*In these initial experiments C8166 cells were used as the susceptible cell line. In all other experiments PM1 cells (which are susceptible to infection by both X4 and R5 variants) were used.

#In these experiments, after a number of day's incubation, the cells appeared rounded and non-adherent. Large patches of the monolayer were decimated and only cell debris was apparent. Transfection efficiencies when calculated were extremely low. Initially it was presumed that the cells were becoming infected by some contaminating organism (i.e. a virus or bacteria). Thus much effort was put into cleaning and thoroughly decontaminating the incubator and other equipment used. Additionally, a number of methods were investigated to ensure that the DNA to be transfected was sterile. Eventually it was determined that the incubator had a major fault in its CO_2 regulation. It is likely that this underlay the continued problems with cell death as 293T cells are particularly sensitive to low (i.e. less than 5%) CO_2 levels. Unfortunately, the CO_2 monitor attached to the incubator continued to give readings of 5% CO_2 when the levels were in fact far lower and thus the

problem remained unrecognised for an extended period of time.

† Shortly after this date a, due to extenuating circumstances, I was assigned a new panel of supervisors and moved to new lab facilities. As a result my work on this project (which requires the use of a containment level three laboratory) was discontinued.

Appendix II: Log of cell separation experiments

Date	Material	Aim	Separation method	Outcome/comments
23/10/01	Marker beads	Generation of calibration curve	Continuous Percoll (20%, 40%, 60% and 80%) gradient at 30,000 and 60, 000 x g	See figure 5.2 30,000 x g gave adequate separation and so all further continuous gradient experiments were carried out using this g force
25/10/01	Whole blood	Attempt separation of cells	Continuous Percoll gradient (5%, 25%, 50% and 60%)	Bands of cells visible
30/10/01	Calf brain	Attempt separation of brain cells	Digestion with 1% collagenase and 784 units/ml papain in 1.5 M NaCl solution at 37°C for 2 hours Continuous Percoll gradient (20%, 40% and 60%)	Thick band of white material (cell debris) visible No other bands of cells visible
30/10/01	Calf brain	To investigate if PBS reduced cell death and clumping	Digestion with collagenase and papain as previous but replacing 1.5 M NaCl with PBS Continuous Percoll gradient (20%, 30, 40% and 70%)	As previous
12/11/01	Human brain	Attempt separation of human brain cells	Digestion with collagenase and papain Continuous Percoll gradient (15%, 25% and 35%) Immunomagnetic separation using MACS beads	Difficult to visualise bands of cells using continuous Percoll gradient MACS separation invalid as cells eluted in negative fraction
18/12/01	Human brain	Attempt separation of brain cells using Ficoll enrichment	Digestion using 10% trypsin in PBS at 37°C for 10 minutes and gentle trituration	Ficoll enrichment successful (see figure 5.3) but large amount of cellular debris in enriched fractions

		followed by continuous Percoll gradient	Ficoll enrichment Continuous Percoll gradient (40%)	Bands of cells poorly visible in continuous gradient- assumed due to clumping with myelin fraction
10/01/02	Human brain	To attempt to reduce cell clumping	As on 18/12/01 but with addition of 2 mM EDTA to all media to reduce clumping of cells	Results as previous
16/01/02	Mouse brain	To investigate effect of storage media on cells propensity to clump	Brain material stored for 0 or 2 days in different media Then procedure carried out as on 18/12/01	All storage methods resulted in large quantity of matter (cellular debris) at top of gradient Bands of cells very faintly visible
15/04/02	Human brain	To attempt immunomagn etic brain cell separation (MACS)	Digestion as above Immunomagnetic separation with MACS beads	Results inconclusive
18/06/02	Human brain	To attempt immunomagn etic brain cell separation (MACS)	Digestion as above Immunomagnetic separation with MACS beads	All cells emerged as positive fraction- cellular debris too sticky
19/06/02	Human brain	To investigate if depletion of debris improves cell separation	Digestion as above Ficoll separation to remove debris Immunomagnetic separation with MACS beads	All cells emerged as positive fraction
1/07/02	Human brain	To investigate if cells stick in absence of antibody and/or beads	Digestion as above Ficoll separation to remove debris Immunomagnetic separation with MACS beads	Cells emerged in "positive" fraction even when no antibody and/or beads included Cells are becoming lodged in matrix probably due to size and/or "stickiness" MACS cannot be used

6/08/02	Human brain	To attempt immunomagnetic brain cell separation (Dyna)	Digestion as above Ficoll separation to remove debris Immunomagnetic separation with Dyna beads	Cell population selected by beads Microscopic examination shows cells encircled by beads Immunocytochemical staining inconclusive
21/08/02	Human brain	To attempt FACS analysis of brain cells	FACS separation	Data difficult to analyse-repeat experiment with prior Percoll step to remove debris
29/08/02	Mouse brain	To remove myelin and cell debris from cell suspension	Digestion with trypsin Discontinuous Percoll separation (layers of 30%, 37% and 70%)	Material aspirated from interface between 30% and 37% is a single cell suspension Most myelin and cellular debris found above 30% layer
30/08/02	J774 cell line	To test if CD11b antibody can be used for FACS analysis	FACS analysis using CD11b antibody followed by fixation using 1% paraformaldehyde	Antibody stained cells (see figure 5.12)
5/09/02	J774 cell line and mouse brain	To test if CD11b can be used to stain mouse brain cells	Removal of cell debris using discontinuous Percoll gradient FACS analysis using anti-CD11b antibody followed by fixation using 1% paraformaldehyde	Antibody able to select population of brain cells-presumed to be microglia (see figure 5.13)
10/09/02	Mouse brain	To investigate staining for intracellular antigens	Digestion with trypsin Removal of cell debris using discontinuous Percoll gradient FACS analysis using anti-CD11b antibody followed by fixation using 1%	CD11b staining successful Inconclusive results for intracellular antigen staining

			paraformaldehyde Fixation in 4% paraformaldehyde and permeabilisation using Triton solution followed by staining with anti-CD68 antibody or anti-GFAP antibody	
25/09/02	Mouse brain	To investigate methods of permeabilisation for staining of intra-cellular antigens	Digestion with trypsin Removal of cell debris using discontinuous Percoll gradient Treatment of cells using either paraformaldehyde fixation alone, paraformaldehyde fixation followed by Triton permeabilisation or methanol fixation/permeabilisation FACS analysis following staining with anti-GFAP or anti-CD68 antibodies	Anti-GFAP antibody stained population of cells after each of the cell treatments Optimal staining observed following Triton permeabilisation Methanol fixation caused clumping of cells Anti-CD68 antibody did not stain cells
10/10/02	Mouse brain	To investigate effect of storage on FACS staining	Removal of brain and storage at room temperature for 1 hour and then at 4°C for 2 days Digestion with trypsin Removal of cell debris using discontinuous Percoll gradient Staining with anti-	Staining successful and comparable to using fresh tissue

			CD11b antibody and fixation using 2% paraformaldehyde FACS analysis	
18/10/02	Mouse brain	To investigate cell yield using either Trypsin or liberase/ DNAse treatment	Digestion with trypsin or liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Staining with anti-CD11b antibody and fixation using 2% paraformaldehyde FACS analysis	Cell yield was approximately twice as high following liberase/DNAse treatment compared to Trypsin.
21/10/02	A172 and U373 cell lines	To test anti-GFAP antibody for FACS	Para-formaldehyde fixation followed by Triton permeabilisation Staining with anti-GFAP antibody and FITC conjugated secondary antibody FACS analysis	Little staining of A172 cells U373 cells stained (figure 5.6) Large degree of non-specific binding of secondary antibody Directly conjugated primary antibody would be preferable
8/01/03	U373 cell line	To test directly conjugated anti-GFAP antibody for FACS	Para-formaldehyde fixation followed by Triton permeabilisation Staining with Cy3 conjugated anti-GFAP antibody FACS analysis	Cells stained by antibody (figure 5.7)
21/01/03	Mouse brain	To investigate methanol fixation	Digestion with liberase/DNAse Methanol fixation Removal of cell debris using discontinuous Percoll gradient Staining with Cy3	Methanol fixation caused cells to clump GFAP staining failed Some degree of CD11b staining

			anti-GFAP or FITC anti-CD11b antibodies FACS analysis	
22/01/03	U373 cell line	To investigate ethanol fixation	Ethanol fixation	Cells aggregated into large clumps
23/01/03	U373 cell line	To investigate "gentle ethanol fixation"	Gentle ethanol fixation Staining with anti-GFAP antibody FACS analysis	Cells remained in single cell suspension- very little clumping apparent GFAP staining successful
27/01/03	Mouse brain	To investigate "gentle ethanol fixation" prior to or post CD11b staining	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with Cy3 anti-GFAP or FITC anti-CD11b antibody OR Staining with anti-CD11b antibody followed by gentle ethanol fixation FACS analysis	When cells fixed prior to CD11b staining, there is a large amount of non-specific staining- isotype control staining equal to anti-CD11b staining Fixation after CD11b staining allows specific staining GFAP staining successful following ethanol fixation (figure 5.8)
29/01/03	Mouse brain	To investigate effect of increasing volume of anti-GFAP antibody used for staining	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with anti-GFAP antibody	No improvement in cell staining with five times increase of antibody

			FACS analysis	
12/06/03	Human brain	To investigate gentle ethanol fixation and staining using human brain	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with Cy3 anti-GFAP Staining with FITC anti-CD11b antibody followed by gentle ethanol fixation FACS analysis	GFAP staining successful (figure 5.9) CD11b staining did not work (figure 5.14)
18/06/03	Human brain	To attempt separation of astrocytes from human brain	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with anti-GFAP antibody FACS sorting	Astrocytes separated
11/07/03	J774 cell line	To test if anti-CD11b antibody still functional	Staining with anti-CD11b antibody followed by gentle ethanol fixation FACS analysis	Staining successful Concluded that the anti-CD11b antibody was not effective for staining human brain cells
01/09/03	HIV infected human brain (150)	To attempt separation of astrocytes and microglia from HIV infected brain	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with Cy3	GFAP staining poor although a few cells were collected (figure 5.10) Antibody with alternative fluorochrome would be preferable CD68 staining successful and cell population sorted

			conjugated anti-GFAP Staining with FITC conjugated anti-CD68 antibody followed by gentle ethanol fixation FACS sort	(figure 5.15)
11/11/03	HIV infected human brain (207)	To attempt separation of astrocytes and microglia from HIV infected brain	Digestion with liberase/DNAse Removal of cell debris using discontinuous Percoll gradient Gentle ethanol fixation followed by staining with AlexaFluor 488 conjugated anti-GFAP Staining with FITC conjugated anti-CD68 antibody followed by gentle ethanol fixation FACS sort	Staining with both antibodies successful and cell populations sorted (figure 5.11)

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